Bile resistance mechanisms in *Salmonella enterica*: genetic and molecular analysis

TESIS DOCTORAL

VERÓNICA URDANETA PÁEZ

Departamento de Genética

Universidad de Sevilla

Bile resistance mechanisms in *Salmonella enterica*: genetic and molecular analysis

Memoria elaborada por Verónica Carolina Urdaneta Páez
para optar al grado de Doctor en Biología

Tesis realizada bajo la dirección del Dr. Josep Casadesús Pursals
en el Departamento de Genética de la Facultad de Biología de la
Universidad de Sevilla

Josep Casadesús Pursals

Verónica Urdaneta Páez

Sevilla, 19 de julio de 2016

INDEX

RESUMEN

Este trabajo centra su investigación en las respuestas adaptativas de *Salmonella enterica* frente a situaciones de estrés. *S. enterica* tiene un ciclo de vida complejo y es capaz de sobrevivir y crecer en numerosos ambientes que incluyen, entre otros, una variedad de hospedadores animales así como múltiples entornos naturales. La versatilidad de esta especie bacteriana viene dada por su capacidad de desencadenar una respuesta apropiada a la variedad de señales ambientales que puede encontrar. Muchos de estos ambientes producen estreses a la bacteria en forma de limitación de nutrientes; pH, temperatura y osmolaridad inestables; desecación; exposición a péptidos antimicrobianos, bilis, agentes oxidantes, etc. Nuestra investigación está enfocada en la respuesta de la bacteria frente al estrés generado por exposición a bilis, una secreción digestiva con propiedades antimicrobianas.

Durante su ciclo de infección, *S. enterica* entra en contacto con bilis en el intestino. La bilis es una secreción compleja, que tiene diversas funciones en la fisiología de los mamíferos. Sirve para la dispersión y absorción de lípidos durante la digestión, siendo secretada por la vesícula en el momento de ingestión de alimentos. La bilis también sirve para eliminar el exceso de colesterol y otros productos del metabolismo hepático. Adicionalmente, las sales biliares poseen potentes propiedades antimicrobianas: actúan como detergentes en la membrana lipídica, desnaturalizan proteínas y causan daños en el ADN, destruyendo la homeostasis bacteriana. Debido a estas propiedades, la capacidad de la flora intestinal y los patógenos entéricos para tolerar la bilis es muy importante para su supervivencia. En *Salmonella*, es igualmente importante para la subsecuente colonización del hospedador.

Existen tres grandes mecanismos de resistencia a sales biliares en *S. enterica.* La primera barrera la constituye la envoltura celular, una estructura compleja con propiedades hidrofóbicas frente a múltiples sustancias antibacterianas incluyendo las sales biliares. La mayoría de los componentes de la envolura que proporcionan resistencia a bilis están en la cara externa de la envoltura celular, principalmente en el lipopolisacárido (LPS). Si las sales biliares logran penetrar en el citoplasma de la bacteria, el segundo mecanismo se activa: las bombas de vertido, que expulsan todo tipo de compuestos nocivos de la célula (incluyendo sales biliares, antibióticos, solventes, etc.). El último nivel de

resistencia a sales biliares lo constituyen los mecanismos de evitación de daño a proteínas y a ADN, y los mecanismos de reparación de ADN.

Un fenómeno de gran complejidad que tiene un papel importante en la resistencia a bilis por parte de *S. enterica* es la adaptación. Consiste en el aumento de la resistencia a bilis tras la exposición a una dosis subletal. Dicha resistencia, que permite sobrevivir a concentraciones letales de sales biliares, es reversible y desaparece una vez que la bacteria vuelve a encontrarse en ausencia de bilis. Aunque se conoce que la adaptación implica cambios en la expresión génica, aún sigue siendo un mecanismo poco entendido.

Para identificar las funciones celulares esenciales para la adaptación a bilis, mutantes con fenotipos sensibles a bilis fueron sometidos a ensayos de adaptación. De este modo se observó que la bomba de vertido AcrAB parecía tener un papel esencial en la la adaptación a bilis. Un escrutinio genético en un mutante *acrA* determinó que únicamente el incremento de expresión de diferentes bombas de vertido podía restaurar parcialmente los niveles de resistencia a bilis en dicho mutante.

Estos resultados llevaron a obtener múltiples mutantes carentes de de bombas de vertido y a estudiar su papel en la resistencia a bilis. La conclusión fue que, a pesar de que se ha indicado que existen 5 bombas de vertido que tienen sales biliares como sustrato, únicamente AcrAB era relevante.

Estudios moleculares del proceso de adaptación mostraron que el primer contacto de cultivos de *S. enterica* en fase exponencial con sales biliares (DOC) conlleva un incremento de la expresión de *acrAB*. A su vez, estudios de citometría de flujo demostraron que en esta fase de crecimiento AcrAB permanece activa independientemente de la presencia de DOC. En fase estacionaria, la presencia de DOC no aumenta la expresión de *acrAB* pero activa el vertido por parte de esta bomba. Estas observaciones sugieren que la activación de la expresión de *acrAB* puede ser crucial durante las primeras etapas de adaptación, mientras que en fase estacionaria la actividad sostenida de estas bombas es suficiente para lograr elevados niveles de resistencia a bilis.

Estudios de mecanismos de resistencia a bilis *in vivo,* utilizando ratones BALB/c como modelo de fiebre tifoidea, llevaron a la conclusión de que la colonización de la vesícula

biliar depende principalmente del proceso de adaptación a bilis de *S. enterica.* Esta conclusión se obtuvo al observar que en la gran mayoría de aislados de la vesícula la resistencia a bilis desaparecía una vez que dichos aislados eran cultivados en un medio libre de sales biliares. Sin embargo, también se obtuvieron algunos aislados con resistencia estable, que fueron considerados mutantes. Los genomas de algunos de dichos aislados fueron secuenciados. Los resultados confirman que la envoltura celular bacteriana es la principal estructura mediadora de resistencia a bilis en *S. enterica:* la mayoría de aislados presentaban mutaciones en genes relacionados con las membranas, la división celular, el transporte, la síntesis de LPS, etc.

Debido a las implicaciones médicas que puede tener la resistencia a bilis de *S. enterica*, el estudio de los mecanismos de interacción bacteria-bilis presentado en esta Tesis puede contribuir a desarrollar nuevos enfoques terapéuticos para erradicar la infección crónica de la vesícula biliar por parte de *Salmonella*, que constituye un serio problema sanitario en determinadas regiones del planeta.

INTRODUCTION

The genus *Salmonella*

The genus *Salmonella* belongs to the *Enterobacteriaceae* family and includes facultative anaerobic, non-spore forming, rod-shaped Gram-negative bacteria. Most Salmonellae are motile and are able to infect a wide variety of animal hosts including reptiles, mammals, birds and amphibians. *Salmonella* is a close relative of *Escherichia*, *Shigella*, and *Citrobacter.*

The genus *Salmonella* is currently divided into two species, *Salmonella enterica* and *Salmonella bongori* (Tindall, Grimont, Garrity, & Euzéby, 2005). *Salmonella enterica* is subdivided within six subspecies [2]: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI). Historically, *Salmonella enterica* subsp. V was *bongori*, which is now considered a different species.

Salmonella subspecies are classified into serovars based on the White-Kauffman classification scheme [3], which relies on antisera that recognize two highly variable surface antigens, O (lipopolysaccharide O-antigen) and H (flagellar proteins) [2,4]. There are more than 2,500 *Salmonella* serovars, most of which belong to the subsp. *enterica* [5]. Serovars of this subspecies regularly colonize warm-blooded vertebrates [6], and they account for 99% of human infections by *Salmonella*. On the other hand, serovars of *Salmonella bongori* and the rest of *Salmonella enterica* subspecies are usually associated to cold-blooded vertebrates or to the environment (Bäumler, Tsolis, Ficht, & Adams, 1998).

Serovars belonging to subsp. *enterica* differ in their host specificity and in the types of diseases they produce. Some serovars are host-restricted, while others can infect a wide variety of animal hosts [8]. The diseases caused by subsp. *enterica* serovars vary from self-limiting gastroenteritis to life-threatening systemic infection, and the outcome of the disease depends on the specific serovar-host combination. For example, the specialist human-restricted serovar Typhi produces typhoid fever, whereas the generalist serovar Typhimurium produces mild gastroenteritis in humans but causes a systemic infection similar to human typhoid fever when infecting mice [9]. For this reason, the interaction between serovar Typhimurium and mice has been extensively used as a model for

typhoid fever in humans [10], and most studies in *Salmonella* have employed this serovar. In this work, we have used the mouse-virulent strain *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344, from the B.A.D. Stocker collection.

According to reports from the World Health Organization (2013), salmonellosis is one of the most common and widely distributed foodborne diseases, with tens of millions of human cases (from which more than a hundred thousand result in death) occurring worldwide every year. In addition, since the beginning of the 1990s, *Salmonella* strains that are resistant to a range of antimicrobials have emerged, and are now a serious public health concern. Furthermore, *Salmonella* is a ubiquitous and resilient bacterium that can survive several weeks in a dry environment and several months in water (WHO, Fact sheet N°139, 2013).

Evolution of *Salmonella* pathogenicity

The genera *Salmonella* and *Escherichia* diverged about 120-160 million years ago [11]. Almost 25% of the *Salmonella* genome consists of genetic material that is absent in *Escherichia coli* [6,12]. The evolution of *Salmonella* pathogenicity is related to the acquisition of virulence factors: the *Salmonella* pathogenicity islands (SPIs) [13,14], which are arranged as clusters of virulence genes in the chromosome. More than 10 SPIs have been described [15], including some which are serotype-specific. The fact the these regions are absent in the chromosome of other Enterobacteriaceae and have different G+C contents than the average of the *Salmonella* chromosome suggests that they have been acquired by horizontal gene transfer (**Figure I1**) [12,14].

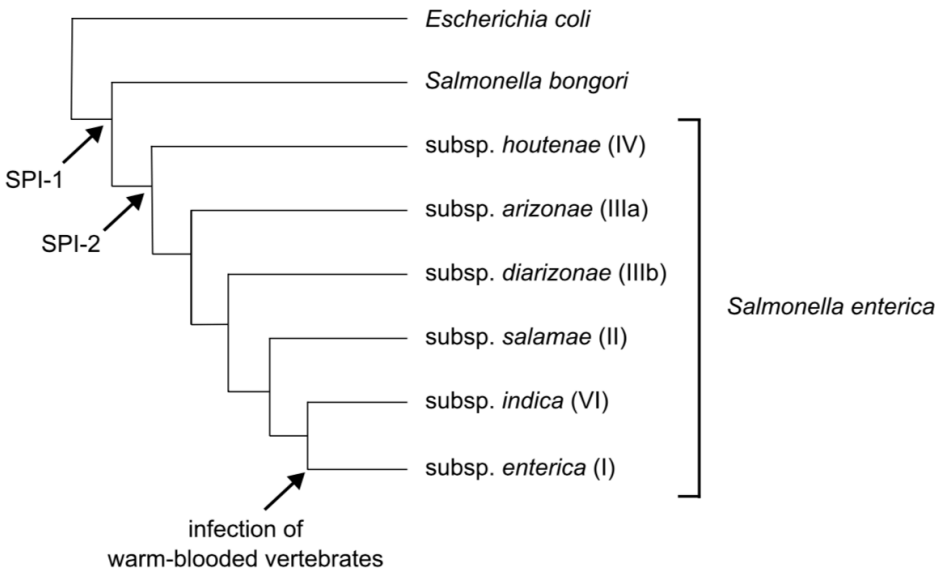

Figure I1. Phylogeny of the genus *Salmonella.* The acquisition of SPI-1, SPI-2, and the ability to infect warm-blooded vertebrates is indicated. Modified from Ellermeier et al., 2006 [16].

The best characterized SPIs are *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2). SPI-1 was acquired first by the common ancestor of the two *Salmonella* species, and is involved in the invasion of intestinal epithelial cells in the animal host [17]. SPI-1 acquisition likely allowed *Salmonella* to become an intracellular pathogen associated with cold-blooded vertebrates [16]. SPI-2 allows *Salmonella* to survive in macrophages and to colonize deeper tissues [18], and its acquisition marked the split of the two *Salmonella* species [16]. Hence, only members of *Salmonella enterica* have the ability to reach deep tissues and organs to produce systemic infections.

The ancestor of subsp. *enterica* acquired the capacity to infect warm-blooded vertebrates, and different lineages subsequently evolved to colonize a variety of hosts. Even though the mechanisms of host specificity are not fully understood, the presence of a virulence plasmid in some serovars of subsp. *enterica* has suggested the potential involvement of plasmid functions [7]. Another factor that may be involved in host specificity is the presence of different sets of fimbrial operons in different serovars [7,19].

Salmonella infection

The *Salmonella* infection process usually begins with the ingestion of contaminated water or food. Figure I2 depicts the biology of the infection by *Salmonella* in humans. The bacteria are capable to survive acid pH in the stomach and to evade the defense systems that it encounters in the small intestine until it reaches the intestinal epithelium. Invasion of this epithelium occurs generally trough M cells, allowing the bacteria to reach lymphocytes B and T which are bellow Peyer patches [20]. Translocation across the intestinal epithelium is mediated by the virulence-associated type 3 secretion system encoded by *Salmonella* pathogenicity island 1 (SPI-1) [21].

Once the epithelium is crossed, *S. enterica* can produce three main types of infection: gastroenteritis, systemic infection and asymptomatic chronic carriage.

In gastroenteritis, non-typhoid strains produce an localized inflammatory response that induces infiltration of polymorphonuclear leukocytes (PMN) into the intestine lumen, and diarrhea by secretion of fluids and electrolytes in the small and large intestines (Figure I2) [20,22].

Systemic infection occurs when bacterial serotypes invade intestinal macrophages and disseminate inside the organism trough the lymphatic system, permitting colonization of internal organs (liver, spleen, bone marrow, and gallbladder) (Figure I2). Within the macrophages, *S. enterica* cells activate virulence mechanisms to survive and replicate in the intracellular environment. In this phase, the type 3 secretion system encoded by *Salmonella* pathogenicity island 2 (SPI-2) plays a crucial role [20,23].

Following systemic infection, a fraction of individuals can become chronic, asymptomatic *Salmonella* carriers, and consequenctly reservoirs for future infections. The mesenteric lymph nodes, the liver and the gallbladder have been proposed as potential *Salmonella* reservoirs [24]. Particularly, colonization of the gallbladder by *Salmonella* in asymptomatic *Salmonella* carriers permits constant shedding of bacteria into the medium since *Salmonella* cells are released into the small intestine each time

the gallbladder contracts. From the small intestine, the bacteria travel downwards in the gut, ultimately being released inside the host feces [20,25].

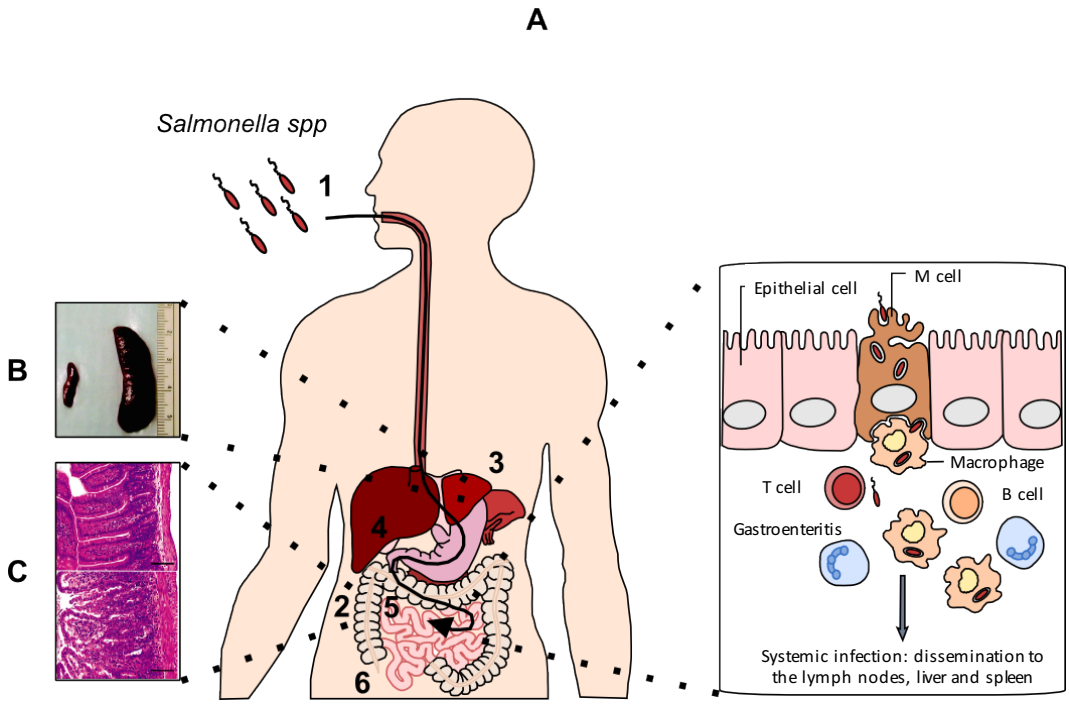

Figure I2. *S. enterica* infection pathogenesis. (A) In humans, typhoid fever is caused by ingestion of food or water contaminated with *Salmonella enterica* serovar Typhi: (1) the surviving bacteria go through the acid pH in the stomach, invade the intestine epithelial cells and migrate to the lamina propia. (2) In the intestine mucosa, *Salmonella enterica* serovar Typhi is phagocytized by macrophages and survives within these cells due to virulence factors, which interfere with the host cells functions. After the invasion, the bacteria express other factors that inhibit detection by the host's innate immune system. This allows the systemic dissemination of the bacteria, colonizing macrophages in the liver, spleen and bone marrow. (3) From the liver, the bacteria can reach the gallbladder, this infection can give rise to a state of *Salmonella* asymptomatic carrier. (4) The *Salmonella enterica* serovar Typhi carriers are continuously secreting bacteria from the gallbladder to the small intestine with the bile, (5) excreting viable bacteria in their feces, (6) consequently infecting other hosts. Image adapted from Tischler and Mckinney, 2010 [25]. (B) Spleen of a non-infected 129sv mouse (left) and the spleen of a 129sv mouse infected with *Salmonella enterica* serovar Typhimurium SL1344 60 days post-infection (right). Image adapted from Monack et al., 2004 [26]. (C) Hystologic sections of BALB/C mice ileum dyed with hematoxilin and eosin (scale: 100 µm). The upper image corresponds to a nonn-infected control, the loweer image shows the ileum of a mouse 5 days post-infection of a lethal dose of *S. Enterica* serovar typhimurium 14028 [10].

In humans, the development of the *S. enterica* chronic carrier state is frequently associated with the presence of gallbladder abnormalities, for instance, gallstones [27]. *Salmonella* is able to form biofilms in gallstones, a matrix that provides high resistance

to antimicrobial agents [28]: typically, antibiotic therapy is ineffective in carriers of *S. enterica* serovar Typhi who suffer of gallstone formation [29].

Salmonella passage through the host: micro-environment conditions and stress responses

As described above, the cycle of infection of *S. enterica* involves a variety of environments, each with different conditions that are far from optimal for bacterial growth and proliferation. Hence, as a foodborne pathogen, *S. enterica* must resist or evade multiple levels of defense to survive within the host. For this purpose, it is essential that the bacterial cells are able to sense and respond rapidly and appropriately to the vast array of stresses they may encounter.

After entering the host orally, *S. enteric* encounters the acidic pH of the stomach. Immediately after, survivors reaching the intestine must face reduced oxygen concentration, bile salts, antimicrobial peptides, weak acids from metabolic products of resident microbial flora, increased osmolarity, and competition with resident microorganisms for nutrients and space [30]. Furthermore, serovars able to adapt and survive these conditions must endure, during epithelium colonization, nutrient depletion within the M cells. Subsequent release into the intestinal submucosa is followed by phagocytosis by resident macrophages allowing the bacteria to escape host humoral defenses. Within the macrophage, the salmonellae reside within phagosomes or possibly phagolysosomes, which present many dangers to the invading bacteria (e.g., acidification, nutrient limitation, generation of reactive oxygen and nitrogen species, and exposure to various antimicrobial peptides (e.g., defensins). Infected macrophages act as vehicles for dissemination of the bacteria throughout the host, causing a systemic infection [31,32].

A relevant consequence of systemic infection is that *S. enterica* reaches and colonizes the gallbladder, a highly adverse environment for bacterial survival: bile, a detergent-like compound with high antimicrobial activity, is concentrated in this organ. Further ahead, we will focus on the strategies that *S. enterica* employs to survive in presence of this substance.

Table I1 simplifies different environmental stresses *Salmonella* endures during the stages of host infection and intestine colonization, and the primary stress responses that *Salmonella* activates to survive.

Table I1. Different stresses experienced by *Salmonella* when colonizing a susceptible host, and main genetic loci that contribute to the response of the bacteria to tolerate this stresses. Compiled from Rychlik and Barrow, 2005; Alvarez-Ordonoez et al., 2016 and Spector and Kenyon, 2011 [30,33,34].

Environment	Stress factor	Regulons induced	Result
Before entering the host	Cold Low nutrients	*csp* *rpoS, rpoE, cyaA, crp.*	General stress resistance. Activation of the starvation-stress response (SSR)
Stomach	Extreme acid pH	*rpoS, fur, phoPQ, ompR, adiA, cadCBA.*	General stress resistance. Induction of acid tolerance proteins. Decrease of intracellular pH. Activation of the acid tolerance response (ATR) and acid resistance mechanisms (AR).
Duodenum	Bile	*phoPQ, oxyR, soxRS, rec, wecD, wecA, acrAB, tolC*	Membrane modifications, invasion suppressed, SOS response, DNA repair, efflux activation, adaptation.
Ileum	Decreased O_2 supply Commensal bacteria: Short chain fatty acids (SCFAs), Bacteriocins, Competitive flora quorum sensing	*frn, arcAB* *rpoS, sdiA, luxS*	Switch from aerobiosis to anaerobiosis, defense against reactive oxygen and nitrogen intermediates. Acid induced resistance to SCFAs. Sensing of AI-1. production of AI-2. Starvation stress response.

Epithelium	Cationic antimicrobial peptides (CAMPs)	*phoPQ, rpoE*	LPS modifications, resistance to macrophage CAMPs.
After leaving the host	Cold shock Low nutrients Aerobiosis	*csp, rpoS* *rpoS, rpoE, cyaA, crp* *arcAB, fnr, oxyR, soxRS*	General stress response Activation of the starvation-stress response (SSR) Switch from anaerobiosis to aerobiosis, Protection against oxidative stress

These stress responses generate a resistant state that allows survival and persistence in the different and changing host environments. Thus, these stress responses and the survival strategies associated with them can have a profound impact on the epidemiology and pathogenesis of these bacteria. We will focus on the stress generated by bile and the strategies that *Salmonella* employs to endure with such conditions.

Salmonella and bile

Bile

Bile is a thick, bitter, yellow-green fluid synthesized in the liver by the hepatocytes. In humans, its daily production is approximately 0.5 to 1 liter, and its pH is between 6.5 and 8 [35]. From the hepatocytes, bile is transported throughout the bile duct to the gallbladder, where bile thickening takes place.

Bile serves as a digestive and as a secretory compound. Although bile does not contain digestive enzymes, it plays an essential role in lipid digestion and absorption: bile is released into the duodenum after food intake, where bile acids, which are potent digestive surfactants, promote lipid absorption [33]. This occurs as acidic and partially digested fats stimulate the secretion of the enteric hormone cholecystokinin, which induces contraction of the gallbladder and the discharge of bile. In addition, bile acts as an excretory agent of many substances and metabolites that cannot be eliminated

efficiently in urine because they are insoluble or protein bound like cholesterol, bilirubin, heavy metals, lipophilic steroids and drug metabolites [35–39].

Bile is composed by both organic and inorganic compounds. 97% of bile is water, but it also has bile salts, inorganic ions (Na^+, Cl^-, HCO_3^-), cholesterol, phospholipids and bile pigments (biliverdin). This fluid is rich in lipids and poor in proteins. Aside from liver isoenzymes of alkaline phosphatase, it does not contain enzymes. Cholesterol solubility is improved by phospholipids and bile salts through the formation of micelles [33,35,36].

Bile salts are produced in the liver from cholesterol, specifically in the pericentral hepatocytes. Bile salts serve as a cholesterol degradation products, and their daily production is approximately 350 mg. Bile salts have predominantly 24 carbon atoms and a complete steroid core [40]. Seventeen different enzymes convert cholesterol into bile acids, which are transformed into bile salts and the association of Na^+ or K^+ ions (this also takes place in the liver). There are four types of bile salts: primary and secondary, conjugated and nonconjugated [35,41]. Conjugation involves the formation of an amide bond with either taurine or glycine.

Primary bile salts are the immediate products of the conversion of cholesterol into bile acids and their structure varies widely between vertebrate species. For instance, in humans and rats, the primary bile salts are cholate and chenodeoxycholate; while in mice they are cholate and muricholate [41].

Secondary bile salts are produced from primary bile salts by intestinal bacteria by removal of the hydroxyl group at C-7. These secondary salts are absorbed from the colon and recirculate with the primary bile salts. In humans, secondary bile salts are deoxycholate (DOC, from cholate) and litocholate (from chenodeoxycholate) [36,42].

Although other bile acids are present in human bile in trace proportions [43], deoxycholate and litocholate compose >95% of all biliary bile acids [38]. The chemical diversity of the bile acid pool is further expanded by enzymatic actions of anaerobic bacteria in the gut, which convert primary bile acids into dozens of secondary and tertiary bile acids [44]. The variety of bile acids in the enterohepatic circulation ensures complete solubilization of hydrophobic nutrients in the small intestine [41].

Figure I3 shows the process of production of the most abundant bile salts present in human bile, along with their chemical structure.

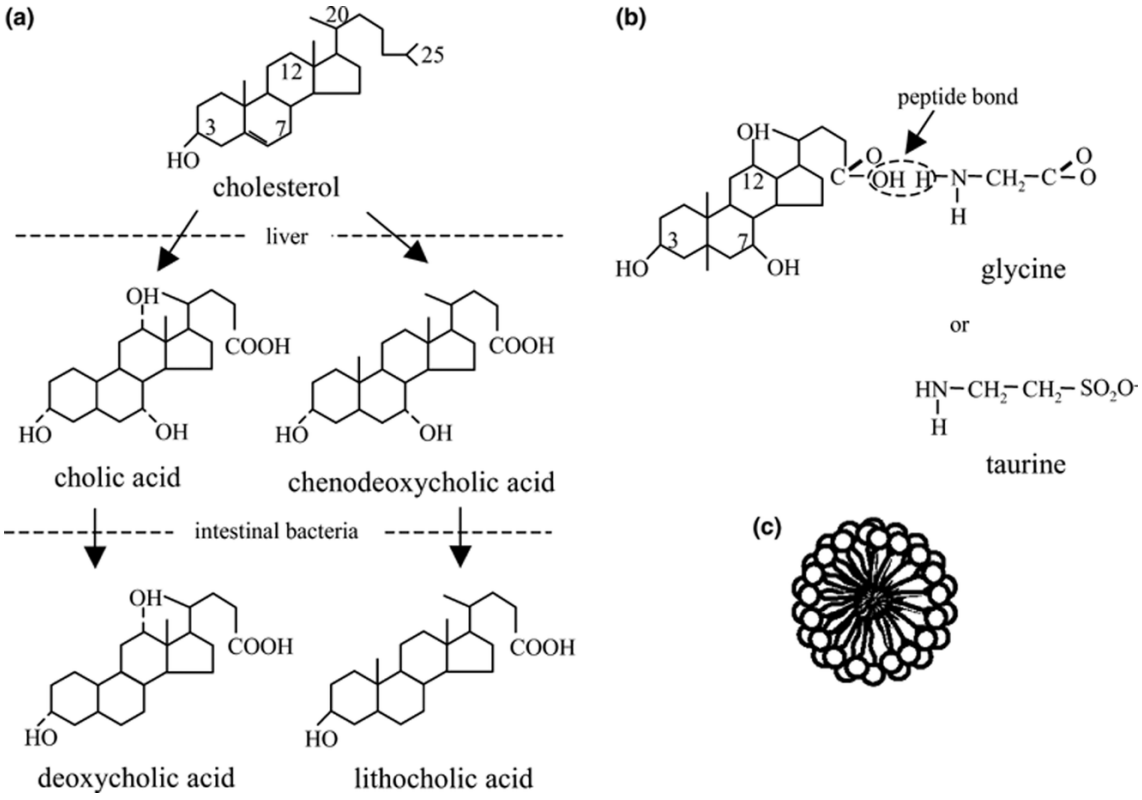

Figure I3. (a) Chemical structure of the major bile acids of human bile. Primary bile acids (cholic acid and chenodeoxycholic acid) are synthesized in the liver from cholesterol; these can be modified by bacterial enzymes in the intestine to form secondary bile acids (deoxycholic acid and lithocholic acid). **(b) All bile acids are conjugated with either glycine or taurine before secretion.** The carboxyl group of the bile acid and the amino group of the amino acid are linked by an amide bond (peptide linkage). **(c) Bile acids are amphipathic and can self-associate to form polymolecular aggregates termed micelles.** These micelles can solubilize other lipids in the form of mixed micelles. Reproduced from Begley et al., 2005 [38].

Bile salts are subjected to a process called enterohepatic circulation: after bile has been released into the intestine and fat digestion and absorption takes place, approximately 95% of bile salts are re-absorbed through high-affinity active transport or by simple diffusion in the distal ileum. Upon entering the blood stream, bile salts are complexed with plasma proteins and returned to the liver. In the liver, bile salts are actively transported into the hepatocytes and secreted again as components of bile [43]. Ileal bile transport is highly efficient (about 95%). Only between 0.3-0.5 mg of bile salts are lost in stools as a payoff the the elimination of excessive body cholesterol [35,43]. Figure I4

shows a diagram of the general process of bile production as well as bile circulation throughout the intestine, the liver, and the gallbladder.

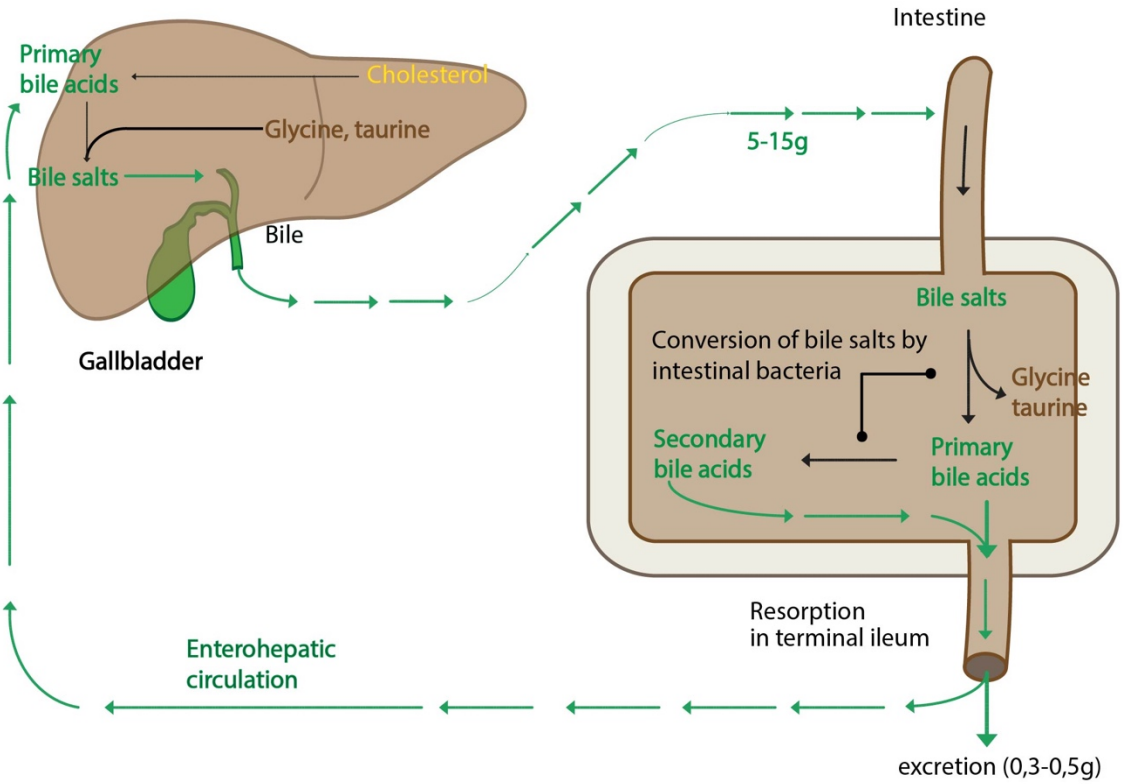

Figure I4. Bile synthesis and circulation in the human hepatic, biliary and digestive system. Bile acids are produced in the hepatocytes from cholesterol, they are then conjugated with taurine or glycine to form primary bile salts (unlike bile acids, these compounds are soluble over a wider range of pH values). Bile salts are concentrated in the gallbladder and, after food ingestion are released into the small intestine. In the intestine, bile salts are further modified by the effect of intestinal microbiota (secondary bile salts). After the digestion, absorption and solubilization and digestion of dietary lipids and lipid-soluble vitamins; bile is reabsorbed in the terminal ileum into de blood stream, and enters the enterohepatic circulation, through which bile salts are returned to the gallbladder [38,43]. Reproduced from Fontana et al., online book [35].

Bile as an antimicrobial agent

The three main bactericidal agents produced by the gastrointestinal system are gastric secretions, hydrochloric acid, and bile. Gastric secretions and hydrochloric acid together lower the pH of the stomach to approximately 3.0. This acidic environment destroys the majority of bacteria that enter the stomach [45]. Surviving bacteria must then face the

harmful effects of bile present in the intestine. As mentioned before, the solubilization of dietary lipids for digestion and absorption is not the only role of bile. Previous studies have described that bile salts inhibit the growth of bacteria in the small intestine [46]; therefore, bile has a second role as a natural antimicrobial compound. The main effects of bile salts on bacteria are the following:

- *Disruption of bacterial cell membranes.*

Bile acids are surface active, amphipathic molecules, and therefore have detergent activity [47]. A comprehensive review [38] reports that the majority of genetic loci disrupted in bile-sensitive mutants are associated directly or indirectly with the maintenance of membrane integrity. Likewise, electron microscopy studies have described a shrunken empty phenotype in Propionibacterium freudenreichii exposed to bile [48] and enzymatic assays in Escherichia coli, Clostridium perfringens and Lactobacillus acidoplilus have shown that bile causes leakage of intracellular material [49,50]. These independent observations provide evidence that bile salts alter cell membrane integrity and permeability. Factors that influence the degree of disruption that bile can have on the cell membrane are as follows: (1) Concentration: high concentrations will dissolve membrane lipids, causing cell leakage and death [51], while low concentrations of bile may have more subtle effects on membrane fluidity and permeability upon alteration of membrane-bound proteins, increase of transmembrane divalent cation flux, and hydrophobicity [49,50,52,53]. (2) The type and structure of bile salts generating the stress. For instance, hydrophobicity is related to the capacity of bile salts to bind membranes: bile salts conjugated with taurine or glycine are fully ionized at physiological pH and for this reason they remain in the outher hemileafelt of the membrane; on the contrary, unconjugated bile salts passively cross membranes and enter the cell [54–56]. (3) Membrane architecture and composition: it has been shown that changes in cell membrane lipopolysaccharides, membrane electric charge, hydrophobicity, lipid fluidity, and fatty acid composition alter bile resistance levels in multiple bacterial species (E.coli, Listeria monocytogenes, Lactobacillus acidophilus) [57–60].

- *Induction of macromolecular instability: secondary structure formation in RNA and DNA damage.*

In *E. coli,* chenodeoxycholate and deoxycholate increase expression of SOS response related genes (*sulA, dinD, osmY, micF*). Increased transcription levels of *osmY* and *micF* genes may suggest that oxidative DNA damage occurs following exposure to bile [61,62]. In *S. enterica,* bile increases the frequency of nucleotide substitutions, frameshifts and chromosomal rearrangements [63], and the mutational spectrum of bile suggests that one primary lesion may be oxidative damage of cytosine [64].

- *Misfolding and/or denaturation of proteins.*

The detergent activity of bile salts can also alter the conformation of proteins. This view is supported by the the observation that bile induces protein chaperones DnaK and GroESL, which are general stress response proteins, as well as the DnaKJ chaperone complex that targets misfolded polypeptides to assist proper protein folding and the GroESL chaperone required for proper folding of novel proteins along with other housekeeping functions [38,65–67].

Bile resistance mechanisms in *Salmonella enterica*

In response of the detrimental effects of bile, enteric commensals and pathogens have developed resistance mechanisms that permit survival. In fact, the ability to resist bile have been used in clinical microbiology to identify enteric bacteria (for instance, Mac Conkey agar) [39]. The cell functions known to contribute to bile resistance in *Salmonella enterica* are listed in Table I2 [33].

Gene or protein	Function of encoded protein	Link to bile tolerance	Reference(s)
phoPQ	Two-component system	*phoPQ* mutant is sensitive to bile	van Velkinburgh & Gunn (1999); Langridge *et al.* (2009)
marRAB	Regulatory genes	*marRAB* are upregulated by bile, *mar* mutants are sensitive to bile	Prouty *et al.* (2004b)
acrAB	Efflux pump	*acrAB* mutant is sensitive to bile, *acrAB* are upregulated by bile	Lacroix *et al.* (1996); Prouty *et al.* (2004b); Langridge *et al.* (2009)
tolQRA, tolC	Efflux pump function	*tol* mutants are sensitive to bile	Prouty *et al.* (2002a); Langridge *et al.* (2009)
dam	DNA adenine methylase	*dam* mutants are sensitive to bile	Heithoff *et al.* (2001); López-Garrido *et al.* (2010); Prieto *et al.* (2004); Langridge *et al.* (2009)
wecD, wecA	Biosynthesis and assembly of enterobacterial common antigen	*wecA* and *wecD* mutants are sensitive to bile	Prouty *et al.* (2002a)
xthA and *nfo*	Exonuclease and endonuclease, respectively, involved in DNA repair	Mutant lacking both *xthA* and *nfo* is sensitive to bile	Prieto *et al.* (2006)
recA, B, C, D, J	Repair and maintenance of DNA	*rec* mutants are sensitive to bile	Prieto *et al.* (2006)
dinB	DNA repair	*dinB* mutant is bile-sensitive	Prieto *et al.* (2006)
seqA	GATC-binding protein	*seqA* mutant is bile-sensitive	Prieto *et al.* (2007); Langridge *et al.* (2009)
hupA	DNA-binding protein	*hupA* mutant is bile-sensitive	Langridge *et al.* (2009)
mrcA, mrcB	Penicillin-binding proteins 1a and 1b	*mrcA, mrcB* mutants are bile-sensitive	Langridge *et al.* (2009)
sanA	Uncharacterized membrane protein	*sanA* mutant is bile-sensitive	Langridge *et al.* (2009)
sbcB	Exonuclease, involved in DNA repair	*sbcB* is upregulated by bile	Prieto *et al.* (2006)
YciF	Unknown function	YciF expression increases in the presence of bile	Prouty *et al.* (2004a)
STM4242	Unknown function	STM4242 expression increases in the presence of bile	Prouty *et al.* (2004a)

Table I2. Genetic loci that contribute to *Salmonella* bile tolerance. How bile salts mediate their antimicrobial activities is still not fully understood; but the effects of certain bile salts can be deduced based on mutations that result in bile sensitivity. Reproduced from Alvarez-Ordonez et al., 2016 [33].

- *Bacterial cell envelope*

The cell envelope of Gram-negative bacteria can be divided in three layers: the cytoplasmic or inner membrane, the peptidoglycan cell wall, and the outer membrane. The outer membrane is highly asymmetrical: its inner leaflet consists mainly of phospholipids, while the outer leaflet is almost entirely composed of a particular kind of glycolipid known as lipopolysaccharide (LPS) [68].

The outer membrane is an excellent hydrophobic barrier to membrane-active agents such as bile salts. Several structures in the cell envelope have been described to play a role in bile resistance.

The lipopolysaccharide (LPS) is an array of large molecules that constitute one of the mayor components present in the outmost leaflet of the outer membrane of Gram-negative bacteria [47]. The LPS can be divided in three structural regions: lipid A (endotoxin), a highly conserved hydrophobic molecule which serves as an anchor to the

membrane; the core saccharide, a genus-conserved short oligosaccharide; and the O-antigen, an immunogenic molecule made up by a number of repeats of the same saccharide unit composed of three to five sugars [69,70]. Most of the structural diversity of LPS is found in the O-antigen. Altered sugar composition, linkage, and number of O-antigen repeats lead to the production of many different kinds of O-antigen molecules both between and within bacterial strains. The O-antigen protrudes from the surface of the membrane and provides the major barrier to external compounds. Loss of the O-antigen results in decreased resistance to bile [47,71]; on the contrary, very long chains have been described to increase bile resistance [72].

Another cell envelope component that contributes to bile resistance in Salmonella enterica bacteria is the enterobacterial common antigen (ECA), a glycolipid different from LPS present in the outer membrane. As a consequence, mutations in genes involved in ECA synthesis (*wecD* and *wecA*), cause bile sensitivity [73].

It has been suggested that envelope barriers are the main defense of *S. enterica* against bile, preventing passage of bile salts through the outer membrane [30]. However, bile salts can enter the cell by crossing the outer membrane (if they are lipophilic uncharged forms) of by passage through porins like OmpF. Once bile enters the cell, additional mechanisms are activated [47,74].

- *Efflux pumps*

These systems mediate the export of bile salts from the bacterial cytoplasm directly out of the cell (which is one of the best-characterized mechanism of bile salt resistance). Efflux systems are responsible for resistance to numerous compounds including antibiotics, oxidative stress agents, organic solvents, and bile salts [47]. Figure I5 shows the efflux systems characterized in *Salmonella enterica*, classified according to the family they belong (RND, resistance nodulation division; MF, major facilitator superfamily; MATE, multidrug and toxic-compound extrusion; ABC, ATP-binding cassette superfamily) along with the main substrates each one exports.

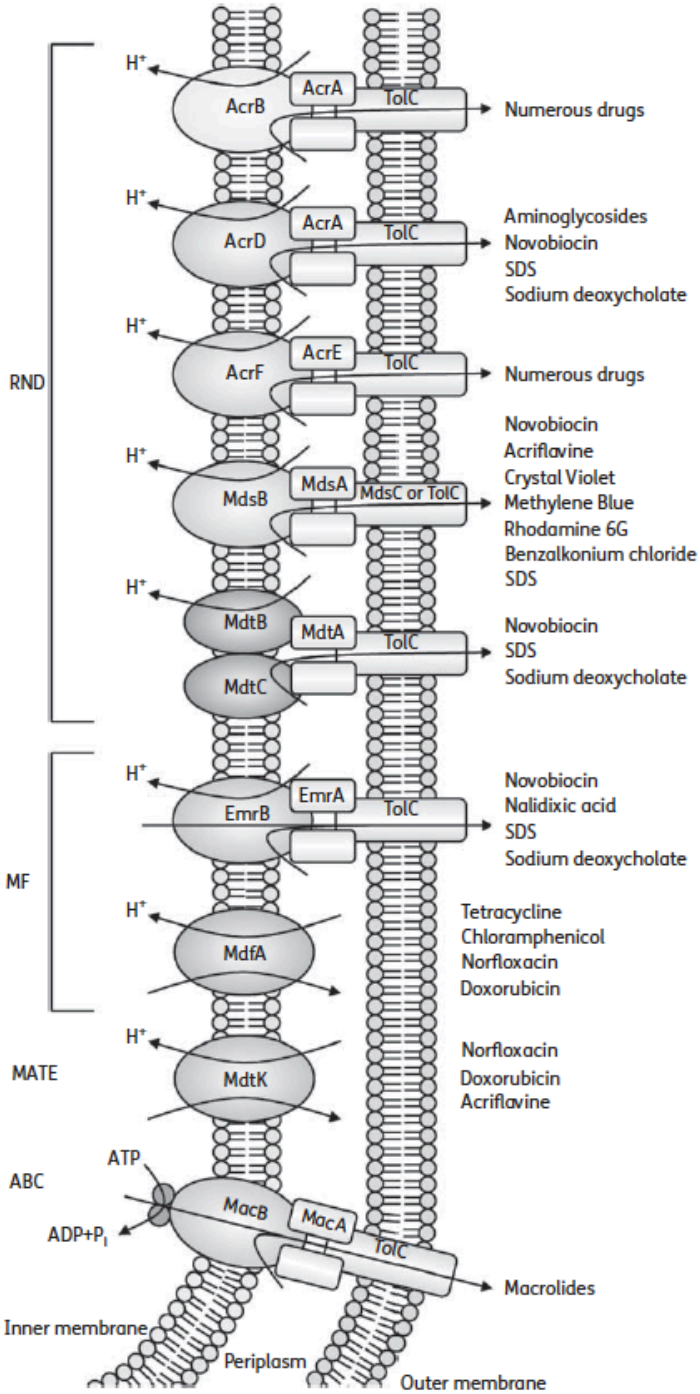

Figure I5. Multidrug efflux systems in Salmonella enterica. Diagrammatic representation of the structure of efflux systems and their location on the membrane. Antibiotic substrates are also listed for each efflux system. Reproduced from Horiyama et al., 2010 [75].

AcrAB-TolC is the best characterized efflux system in enteric bacteria [76–79]. This efflux pump comprises the outer-membrane channel TolC, the secondary transporter AcrB located in the inner membrane, and the periplasmic AcrA, which bridges these two integral membrane proteins. The AcrAB–TolC efflux pump is able to transport vectorially

a diverse array of compounds with little chemical similarity [80]. This system is necessary for the intrinsic resistance of *E. coli* to solvents, detergents, dyes, lipophilic antibiotics, and bile salts. In *S. enterica*, deletion of the *acrA, acrB* or *tolC* genes confers increased susceptibility to antimicrobial agents and chemical compounds, including bile, [81]. Figure I6 depicts the AcrAB-TolC efflux system structure and location within the bacterial cell membrane.

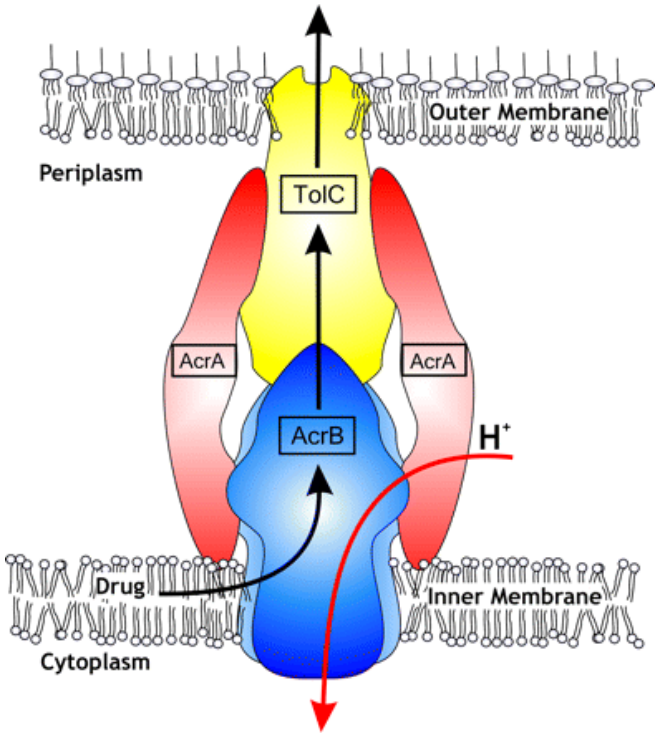

Figure I6. Schematic drawing of the tripartite RND multidrug efflux system AcrAB-TolC in *E. coli.* Suggestions on the stoichiometry of the adaptor AcrA to inner membrane RND component AcrB (or to outer membrane channel TolC) vary between 1 and 4. AcrB is a proton force-dependent drug efflux transporter, and AcrA is thought to aid in direct cytoplasm to environment efflux by bridging the inner and outer membranes. An outer membrane protein, TolC, contributes to the function of the Acr system. Reproduced from Pos, 2009 [83].

The genes encoding the AcrAB-TolC multidrug efflux system are under the control of a transcriptional regulator known as RamA. In turn, the *ramA* gene is transcriptionally activated by bile, mainly by inhibiting transcriptional repression exerted by RamR [79,84].

- *DNA repair mechanisms*

DNA adenine methylase (*dam*) mutants of *S. enterica* are bile-sensitive [85], and a study

on the causes of this phenotype unveiled the involvement of Dam-directed mismatch repair [63]. Mutations in any of the mismatch repair genes *mutHLS* suppressed bile sensitivity in *dam* mutants, providing evidence that bile sensitivity was caused by MutHLS activity. An analogy between these observations and previous studies in *E. coli* supported the view that DNA strand breakage by the MutHLS system renders *dam* mutants sensitive to bile salts (39). *Salmonella* mutHLS mutants are not sensitive to bile (39), indicating that bile-induced DNA damage can be repaired by mechanisms other than Dam-dependent mismatch repair. In *dam* mutants, however, lack of DNA strand discrimination causes extensive DNA strand breakage when the MutHLS systems deals with bile-induced lesions [63].

Surveys of bile sensitivity among S. enterica DNA repair mutants has revealed that base excision repair (BER), SOS-associated DNA repair and recombinational repair by the RecBCD enzyme are required to cope with bile-induced DNA damage (40). In contrast, nucleotide excision repair (NER) is dispensable. The observation that bile resistance requires BER but not NER suggests that bile-induced DNA lesions are unlikely to be bulky, thus providing indirect support for the oxidative damage hypothesis [63,64].

Several lines of evidence suggest that bile salts may impair DNA replication in Salmonella: (i) *lexA(ind)* and *dinB* mutations confer bile sensitivity, suggesting that SOS-associated translesion DNA synthesis may be required to overcome bile-induced DNA damage (40); (ii) *recB, recC,* and *recA recD* mutants are also bile sensitive, indicating that survival to bile requires RecB-dependent homologous recombination [64]. Primary lesions (e. g., oxidized cytosine moieties) can be expected to trigger base excision repair, and the activity of BER exonucleases will produce DNA strand breaks as an intermediate step in the DNA repair process. Furthermore, as indicated above, DNA strand breaks can also be formed as a consequence of MutHLS activity. These DNA strand breaks may impair progression of DNA replication forks, inducing the SOS response; as a consequence, DinB-mediated translesion synthesis may occur. It is also conceivable that bile-induced lesions could directly block DNA replication, thus inducing the SOS response in a direct fashion. In such a scenario, the need of homologous recombination mediated by the RecBCD enzyme might reflect the occurrence of stalled DNA replication forks [64].

- *Salmonella enterica adaptation to bile*

Salmonella enterica can be adapted to growth in extremely high bile concentrations if previously exposed to sub-lethal doses [86]. This increase in bile resistance levels is transitory: removal of bile from the medium will cause that the levels of resistance return to its original levels. This phenomenon occurs preferentially if the bacteria is exposed to near lethal bile concentrations during logarithmic growth phase [47]. A transcriptomic analysis performed by Prouty *et. al* (2004) in the presence of a sublethal concentration of DOC (3%) identified at least 230 *Salmonella* genes with altered expression (about 3% of the *Salmonella* genome). Of these genes, 101 were activated by bile and 129 were repressed by bile [87]. In turn, a study carried out in our laboratory in the presence of a higher concentration of DOC (5%) revealed also multiple changes in gene expression [88]. The latter study proposed that activation of the RpoS-dependent general stress response plays a crucial role in the adaptation of Salmonella to bile, a view supported by the observation that *rpoS* mutants are bile-sensitive [88]. Adaptation to bile involves a response by the bacterial population, and might occur during the infection process, specifically along the passage of bacteria through the host intestine, where bile is released in low concentrations. This exposure might allow the cell to endure the increasing bile concentrations found in the hepatobiliary tract, and ultimately in the gallbladder. In turn, a study carried out in our laboratory in the presence of a higher concentration of DOC revealed also multiple changes in gene expression [88].

OBJECTIVES

Previous studies of our laboratory had identified cellular functions that contribute to bile resistance in *Salmonella enterica.* Such functions include components of the cellular envelope, stress responses, and DNA repair networks. These studies had also provided evidence that the phenomenon known as "adaptation to bile" (the increase of bile resistance upon exposure to sublethal concentrations) might involve multiple changes in gene expression. This Thesis intends to understand the molecular basis of adaptation of *Salmonella enterica* to bile, using a combination of classical genetics, molecular biology, single cell analysis, and infection of mice. The specific objectives are as follows:

Objective 1. Identification and characterization of *Salmonella enterica* functions involved in adaptation to bile.

Objective 2. Investigation of the roles of *S. enterica* efflux pumps in bile resistance and adaptation.

Objective 3. Characterization of *S. enterica* bile-resistant isolates from the gallbladder of BALB/c mice.

Objective 4. Characterization of bile-resistant mutants of *S. enterica* isolated from the gallbladder of BALB/c mice.

MATERIALS AND METHODS

Bacterial strains

Salmonella enterica serovar Typhimurium strains used in this thesis are listed in Table M1. *Salmonella enterica* strains are derivatives of SL1344; for simplicity, *S. enterica* serovar Typhimurium SL1344 is often abbreviated as *S. enterica.*

Table M1. Table of strains used in this work.

Strain name	Genotype
SV5015	*SL1344 his*$^{+}$ (WT) [1]
SV5367	*Δdam* [2]
SV7248	*acrA::*Km^{R}
SV7458	*acrB::*Km^{R}
SV6279	*asmA::*Km^{R} [3]
SV6544	BR6 [4]
SV6947	*damX::*Km^{R} [3]
SV6934	*phoP::*MudJ (Km^{R}) [3]
SV6246	*prc::*Km^{R} [3]
SV4227	*rpoS::*Ap^{R} [5]
SV6629	*tolC::*Km^{R} [3]
SV4586	*wecD::*MudJ (Km^{R}) [1]
SV6268	*zapB::*Km^{R} [3]
SV6745	*acrD::*Km^{R} [3]
SV7249	*acrF::*Km^{R}
SV7250	*emrA::*Km^{R}
SV7251	*macA::*Km^{R}
SV7252	*mdfA::*Km^{R}
SV7457	*mdsA::*Cm^{R}
SV7253	*mdtA::*Km^{R}
SV7254	*mdtK::*Km^{R}
SV7617	*Δ acrD mdtK*::Km^{R}
SV7620	*Δ acrD mdtK mdfA::*Km^{R}
SV7511	*Δ acrD mdtK mdfA emrA::*Km^{R}
SV7626	*Δ acrD mdtK mdfA emrA acrF::*Km^{R}
SV7631	*Δ acrD mdtK mdfA emrA acrF mdtA::*Km^{R}
SV7634	*Δ acrD mdtK mdfA emrA acrF mdtA, macA::*Km^{R}
SV7636	*Δ acrD mdtK mdfA emrA acrF mdtA, macA::*Km^{R}*, mdsA::*Cm^{R}
SV8204	*ΔacrA, prc::*Km^{R}
SV8205	*ΔacrA, asmA::*Km^{R}

SV8206	BR6, *acrA::*Km^R
SV9079	*ΔMR-MLE*
SV9080	*Δrtn*
SV9081	*ΔsptP*
SV9082	*Δudg*
SV9083	*ΔulaA*
SV9084	*ΔyfbG*
SV9085	*ΔyohI*
SV7762	*ΔleuO* [6]

[1] Constructed by Francisco Ramos Morales
[2] Constructed by Roberto Balbontín
[3] Constructed by Sara Belen Hernández
[4] Described by Hernández *et. al.* 2012 [14]
[5] Constructed by David Cano
[6] Constructed by Elena Espinosa

Bacteriophages

P22 HT105/1 *int201* [89] was employed as transducing bacteriophage. For P22 sensitivity assays, the clear-plaque H5 derivative, which harbors a mutation in *c2* gene [90], was used. The P22 *c2* gene is an equivalent of *cI* gene in phage λ.

Culture media, solutions and growth conditions

Bacterial media and growth conditions

Culture media

• LB: Luria-Bertani medium. Rich medium used for the normal growth of *S. enterica* and *E. coli*:

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l

• Sodium choleate (ox bile extract) medium: Rich medium used to assay *S. enterica* bile resistance levels:

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
Sodium choleate (Sigma-Aldrich)	150-300g/l

• Sodium deoxycholate (DOC) medium: Rich medium used to assay *S. enterica* bile resistance levels:

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
DOC (Sigma-Aldrich)	5-50g/l

• EBU: Rich medium used to discard the presence of lysogenic isolates after transduction. EBU is LB medium supplemented with the following components:

K_2HPO_4 25%	10 ml/l
Glucose 50%	5 ml/l
Fluorescein 1%	2.5 ml/l
Evans Blue 1%	1.25 ml/l

• SOB: Rich medium used to grow competent cells:

Tryptone	20 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l
KCl	0.19 g/l
pH 7	adjusted with NaOH

After autoclaving, 5 ml of $MgCl_2$ 2 M was added.

For solid media, agar was added to a final concentration of 15 g/l. When necessary, antibiotics or other chemicals were added to the medium to the final concentrations shown in Table M2.

Table M2. Final concentrations of antibiotics and other chemicals used in this study.

Chemical	Final concentration
Antibiotics	
Ampicillin (Ap)	100 µg/ml
Chloramphenicol (Cm)	20 µg/ml
Kanamycin (Km)	50 µg/ml
Tetracycline (Tet)	5 µg/ml
Streptomycin (Str)	200 µg/ml
Other chemicals	
EGTA	0.004 µg/ml

Growth conditions

S. enterica cultures were routinely grown at 37° C, and exceptionally at 30° C. Cultures were shaken at 200 rpm. For microaerophilic conditions, 5 ml of bacteria were incubated at 37° C without shaking in 10ml plastic tubes.

Solutions

- PBS 10x:

NaCl	1.37 M
KCl	27 mM
$Na_2HPO_4 \cdot 7H_2O$	43 mM
KH_2PO_4	14 mM
pH 7.3	

Bacterial transduction

P22 lysates

To prepare P22 lysates, 1 ml of P22 stock was mixed with 0.25 ml of the donor strain. The mixture was incubated at 37° C and 200 rpm for 8-16 h (8 h is the optimal time to obtain the highest rates of cellular lysis). Bacterial debris was removed by centrifugation

for 20 min at 4500 rpm. The supernatant was recovered in a fresh tube, and 250 µl of chloroform were added, the mix was then vortexed. The lysates were maintained at room temperature for a few hours and then stored at 4° C; under these conditions the lysates are stable for months or years.

- P22 stock:

NB	100 ml
E50x	2 ml
Glucose 20%	1 ml
P22 phage	0.1 ml

- NB: Nutrient broth

Meat extract	3 g/l
Peptone	5 g/l

- E50x:

$H_3C_6H_5O_7 \cdot H_2O$	300 g/l
$MgSO_4$	14 g/l
$K_2HPO_4 \cdot 3H_2O$	1965 g/l
$NaNH_4HPO_4 \cdot H_2O$	525 g/l

The chemicals were added to 1 l of warm H_2O following the order indicates in the table. Water was added until the total volume reached 3 l. The medium was cooled and then sterilized with chloroform.

Transduction in liquid medium

To carry out transductions in liquid medium, 100 µl of an overnight culture of the recipient strain and 10 µl of the donor strain lysate were mixed in a sterile 1.5 ml tube. This mix was incubated at 37° C and 200 rpm for 30-45 min (depending on the marker of transduction). The mix was then spread on selective medium plates that were incubated at 37° C until colonies appeared.

This method does not yield independent transductants, but incubations shorter than 30 minutes do not permit transductants to divide, so the proportion of twins is minimal. On the other hand, it has the advantages of being fast and repetitive. Using dilutions of the transduction mixture, a number of multiplicities of infection can be tested for the same transduction.

Detection of lysogenic transductants

Transductans harboring a selective marker could have been infected by P22 phage and become pseudolysogenic (the *int* mutation avoids integration, and delays the formation of true lysogens). As time goes on, pseudolysogens become resistant or immune to new P22 infections and cannot be lysed or transduced again. Pseudolysogeny should thus be avoided. For this purpose, transductant colonies were isolated in EBU plates (with antibiotics if necessary). In these plates, pseudolysogens are dark colored and P22-free colonies are light colored. This color difference is due to cell lysis in the pseudolysogenic colony, which causes acidification of the medium and turning of the pH indicator, darkening the agar. A transductant was considered P22-free when streaking did not give rise to any dark colony.

P22 sensitivity assay

In EBU plates, isolates that forms light color colonies could be lysogens that do not undergo visible lysis. These isolates are P22-resistant and can be mistaken by real P22-free isolates. To avoid this situation, an assay to detect P22-sensitive strains is advisable. A streak with a P22 H5 lysate is done on an LB or EBU plate, and air-dried. The test strain is then streaked in a perpendicular way to the H5 streak. P22-sensitive strains grow until they reach the H5 streak, while P22-resistant strains grow over the streak.

DNA manipulation and transfer

Plasmids

Plasmids used in this study are listed in Table M3.

Table M3. List of plasmids used in this thesis.

Plasmid	Description	Reference
pCP20	bla cat cI857 λPR flp pSC101 oriTS, Apr, Cmr	Cherepanov and Wackernagel, 1995 [91]
pKD3	bla FRT cat FRT PS1 PS2 oriR6K, Apr , Cmr	Datsenko and Wanner, 2000 [92]
pKD4	bla FRT aph FRT PS1 PS2 oriR6K, Apr , Kmr	Datsenko and Wanner, 2000 [92]
pKD13	bla FRT aph FRT PS1 PS4 oriR6K, Apr , Kmr	Datsenko and Wanner, 2000 [92]
pKD46	bla PBAD gam bet exo pSC101 oriTS, Apr	Datsenko and Wanner, 2000 [92]
pBR328	Cloning vector Amp^r, Km^r, Cm^r	Bolivar and Backman, 1979 [93]

Extraction of plasmid DNA

For the extraction of plasmid DNA the commercial system GenElute™ Plasmid Miniprep Kit, provided by Sigma-Aldrich Co (St. Louis, Missouri, USA), was used.

Extraction of genomic DNA

For the extraction of genomic DNA, 5 ml of cells grown in exponential phase were collected and re-suspended in 0.4 ml of lysis buffer, 4 µl of RNAse (10 mg/ml) were added and the mixture was incubated at 37° C for 30 minutes. After that, 20 µl of proteinase K (20 mg/ml) was added and the sample was incubated for 2 h at 65° C. Finally, 3 or 4 extractions were performed with phenol:chloroform-isoamyl alcohol in a 2:1 proportion. Optionally, one last extraction with chloroform:isoamyl alcohol (24:1) can be performed. DNA was precipitated at -20° C by adding 1/10 volume of sodium acetate 3 M and 2.5 volumes of ethanol. After precipitation, genomic DNA was washed with 70 % ethanol and re-suspended in 20 µl of TER buffer.

- Buffer lysis

Tris-HCl	50 mM pH 8
EDTA	10 mM
NaCl	100 mM
SDS	0.2 %

- TER:

Tris-HCl	10 mM pH 7.5
EDTA	1 mM pH 8
RNAse	20 µg/ml

Digestion, modification and ligation of DNA fragments

Restriction endonucleases were supplied by Roche Diagnotics GmbH (Indianapolis, Indiana, USA), New England Biolabs (Beverly, Massachusetts, USA) and Promega Biotech (Madison, Wisconsin, USA). In each case, enzymes were used following the manufacturer's instructions.

For ligation of DNA fragments, 1 U of T4 DNA ligase (1U/µl, Roche Diagnostics) was used in the buffer supplied by the manufacturer. Routinely, the mixture was incubated at 16° C for 12 hours at least.

Agarose DNA gel electrophoresis

Electrophoresis in an agarose gel was used to test the quality of DNA extraction, to determine DNA fragments after plasmid restriction, to estimate the efficiency of endonuclease restriction, to confirm PCR amplification, etc. The agarose gel was submerged in TAE 1x buffer.

Low Electro Endosmosis agarose (Pronadisa, Conda, Spain) was employed. Its concentration varied between 0.8 and 1.5 % depending on the size of the fragments to be separated. The loading buffer used was a solution of bromophenol blue (0.125%) and Ficoll 400 (12.5%).

The 1 Kb ladder (GIBCO BRL, Life Technologies, New York, USA) was used as molecular weight marker. Samples were mixed with 1/10 of loading buffer. Ethidium bromide (0.5 µg/ml final concentration) was added to the gels to make bands visible. Gels were illuminated with a UV transilluminator; pictures were taken with a Polaroid ISO3000/36 snapshot film.

- TAE

Tris-acetate	40 mM
EDTA	10 mM pH 7.7

Isolation of DNA fragments from agarose gels

For the isolation of DNA fragments from agarose gels was used the commercial system Wizard® SV Gel and PCR Clean-Up System supplied by Promega Co.

Bacterial transformation

High efficiency *E. coli* transformation

Competent cells were prepared using a variation of the Inoue method [94], which guarantees high transformation efficiency (between $5x10^7$ and $5x10^8$ transformants per µg of plasmid DNA). An overnight culture of *E. coli* DH5α was diluted 100-1000 times in 200 ml of SOB, and incubated at 22° C and 200 rpm until the OD_{600} reached 0.5. The culture was chilled quickly on ice and kept on it for 10 minutes. Cells were harvested by centrifugation at 2500 g and 4° C for 10 min. The pellet was re-suspended in 20 ml of cold TB, and 1.5 ml of DMSO was added. After a 10 min incubation on ice, aliquots of 0.2 ml or 0.5 ml were prepared, frozen in liquid nitrogen, and stored at -80° C.

For transformation, an aliquot of competent cells was slowly thawed on ice and was mixed with the plasmid. The mixture was incubated on ice for 30 minutes, subjected to heat shock (42° C, 45 s), and cooled on ice for 1 min. One ml of LB was then added. The mixture was incubated at 37° C for 1 h; finally, the cells were concentrated in 100 µl and spread on selective media.

- TB

PIPES	10 mM
$CaCl_2$	15 mM
KCl	250 mM
pH 6.7 with KOH	
$MnCl_2$	55 mM

This solution was sterilized by filtration.

Bacterial electroporation

An overnight culture, was diluted 1/100 in LB and, depending on the strain, was grown at 37° C or 30° C until a 0.6-0.8 OD_{600} was reached. The culture was chilled on ice and kept on it for 5 minutes. 25 ml were transferred to a tube, and the cells were harvested by centrifugation at 4000 rpm for 5 min at 4° C. The supernatant was discarded and the bacterial pellet was softly re-suspended in 1 ml of cold ddH_2O. Afterwards, 24 ml of cold water were added; this wash was repeated a second time. Finally, cells were harvested and re-suspended in 250 µl of water.

Electroporation was performed by mixing 1 µl of plasmid DNA or 10 µl of PCR product with 40 µl of competent cells. The mixture was transferred to a cooled cuvette with 2 mm of distance between the plates. The cuvette was subjected to an electric discharge in the electroporator (2.5 KV, 200 Ω and 25 µF). The electroporator employed was a BTX Electrocell Manipulator 600 (Harvard Apparatus, Holliston, Massachusetts, USA). After the discharge, 1 ml of LB was added to the cells, which were then transferred to a 10 ml plastic tube and incubated at 37° C with shaking (200 rpm) for 1 h. Finally, cells were concentrated in 100 µl and spread on selective media.

Genetic screens

T-POP transposon genetic screen

T-POP transposon mutagenesis was done using the strain wt strain SL1344 as the recipient, the transducing phage P22 was used as the vector of choice to insert the transposon into cells, the aim of this procedure was finding bile resistant phenotypes by insertion the T-POP, either by gene inactivation caused by its disruption or by increased expression of the surroundings of the T-POP insertion site [95,96].Nine pools of T-POP3 insertions, which were previously prepared, were used to generate mutagenesis by chromosomal homologous recombination within the recipient strain.

Briefly, to obtain these pools a lysate carrying a T-POP3 (Tet^R) insertion in an F' plasmid was prepared. This lysate was used to transduce a strain (*S. enterica* wild type strain) carrying the pNK2880 plasmid [97]. This plasmid produces ATS Tn10 transposase, allowing T-POP3 transposition into the recipient chromosome. As a result, all tetracycline-resistant mutants isolated will inherit T-POP3 by transposition [96]. ATS transposase is less specific than the wild type transposase of Tn10. During transduction, the transposon enters the receptor strain inserted in a F' fragment, and cannot recombine with the host chromosome because there is no homology.

To obtain an independent collection of insertions of the T-POP3 transposon, the following steps were followed: (i) 10 µl of a T-POP3 (Tet^R) lysate were transduced to 100 µl of the strain harboring pNK2880; (ii) transductions were incubated at 37° C for 30 minutes; (iii) transductions were spread in LB plates and incubated for 4 hours; (iv) replicates were made in LB with Tet and EGTA 10 mM (compound that chelates Ca^{2+} ions and avoids the P22 capsid assembly, by this way, reinfections are avoided and the probability of appearance of pseudolysogenics decreases) and incubated at 37° C overnight; (v) transductant colonies were recovered with 1 ml of LB with EGTA. Colonies of various plates can be mixed; usually, collections are made with 2000-5000 colonies; (vi) 1 ml of cells were frozen with 75 µl of DMSO at -20° C; (vii) collection tubes were thawed in ice and diluted 1:25 in 5 ml of LB with Tet and EGTA; (viii) cells were incubated for 1.5 hours at 37° C, and centrifuged at 13000 rpm for 3 minutes. The pellets were washed 3-4 times with LB to eliminate EGTA; (ix) cells were re- suspended in 1 ml of LB; and (x) a lysate was prepared as described previously.

These P22 phage lysate pools, with independent T-POP3 chromosomal insertions, can be used in subsequent crosses involving cells that do not express transposase, therefore the insertions will be inherited by homologous recombination.

pBR328-based plasmid library genetic screen

A *S. enterica* genomic library cloned into pBR328 plasmid was used to screen bile sensitive phenotype suppressors on the *acrA::Km* strain background (SV7248).

This library was previously constructed by partial digestion of genomic DNA from

Salmonella enterica Serovar Typhimurium SL1344 with Sau3A. DNA fragments 7-11 kb long were ligated to the pBR328 vector, which was previously digested with BamHI and dephophorylated. *Salmonella* strain TR5878 was transformed with the ligation products, and ampicillin-resistant clones were selected on LB Ap plates. Pools of approximately 1000 independent transformants were collected and lysed with phage P22 HT 105/1 *int201*. As a quality control, the ability of the library pools to complement null mutations in *araA* (required for growth with L-arabinose as the sole carbon source) or *xylA* (required for growth with D-xylose as the sole carbon source) was tested. Lysates that permitted successful complementation were stored and used for plasmid delivery to recipient strains in subsequent genetic screens.

The genetic screen was done by mixing 100 µl of a saturated *acrA::Km* culture with 10 µl of each P22 lysate of the pBR328 plasmid library pools. After 45 minutes, the transductions were plated in LB Cm 0.5% DOC agar plates and incubated at 37° C overnight. There were 9 different pools, and 10 transductions were made out of each of the pools (90 transductions total). As a control, 5 additional transductions using as the recipient the wt SL1344 strain were made from aleatory pBR328 pools, they were also plated in LB Cm 0.5% DOC agar plates and incubated at 37° C overnight.

The following day, the *acrA* background colonies obtained were and picked in LB Cm agar plates containing increasing DOC concentrations (from 1 to 7%). The candidates that showed an increased level of DOC resistance were cultured in LB Cm liquid medium, Minipreps were done to recover the pBR328 plasmid derivatives and sent out for DNA sequencing of the inserts.

Construction of bacterial strains

Oligonucleotides

Oligonucleotides used in amplification and sequencing were provided by Invitrogen Life Technologies and are listed in Table M4.

Table M4. Oligonucleotides used in this study.

Oligonucleotide name	Sequence 5'-3'
MR-MLE-P4-FOR	TGTATCACGAACCTGATAAATGGAGAAATTATGAAAA TTAATTCCGGGGATCCGTCGACC
MR-MLE-P1-REV	GCATAAACCATAATCAGTATCACCAGATGCTATTTAAT CGTGTAGGCTGGAGCTGCTTC
MR-MLE-E1	GTTCAAGTCTTGGCGGATTC
MR-MLE-E2	CAATACCTCCCGTGTATACC
rtn-P4-FOR	AATGATTTGAAAGTAAGAAAGCTATTTCTGATGTTGTT TAATTCCGGGGATCCGTCGACC
rtn-P1-REV	TGAATTTTTTTCCGTAGCGAGTTCCTGCATTATTCAGC CGTGTAGGCTGGAGCTGCTTC
rtn-E1	CTAACGATAGTATCCTGCACC
rtn-E2	CAGATATCGTTATCGACTCTCCGG
sptP-P4-FOR	AGGAAGCGCTCAAAAACATACTGCAGGAATATGCTAA AGTATTCCGGGGATCCGTCGACC
sptP-P1-REV	GATAGTTCTAAAAGTAAGCTATGTTTTTATCAGCTTGC CGTGTAGGCTGGAGCTGCTTC
sptP-E1	TCTATCTGGCGGCAGATTATGG
sptP-E2	TTGGCGCTTATAATGCCG
udg-P4-FOR	TCATAATTAAGTTAATTCTGAGAGCGAATAATGAAAA TCAATTCCGGGGATCCGTCGACC
udg-P1-REV	TAAATCATCGGGACTCATCAGCCCCGATTGCCTTATTA AGTGTAGGCTGGAGCTGCTTC
udg-E1	GAATTGTTTTCGTCTGCGTTGCAC
udg-E2	CAATCTGTTCCGGATCGTTC
ulaA-P4-FOR	TAAACAATAATTATTGCTGGCGAGGTGGATATGTTTA TCCATTCCGGGGATCCGTCGACC
ulaA-P1-REV	AATATCGCGCGCCAGTTGGGTGATTTCGGTTACGTTC ATGTGTAGGCTGGAGCTGCTTC
ulaA-E1	AGGTGGAACACTCCGATCTTTC
ulaA-E2	ATGGCCTTTCGACAGTACGAAG
yfbQ-P4-FOR	GTGGCAATAACAGCTAAAAGGTCAAGATTCATGTCCC CCAATTCCGGGGATCCGTCGACC
yfbQ-P1-REV	CAGGCTACCGAAGTAGCCTGAATTTAAGATTATTGAT GGGTGTAGGCTGGAGCTGCTTC
yfbQ-E1	AACTCACCGTCGTGGGAGTAC
yfbQ-E2	TGCAGACTATGTTCGGAGAC

yohI-P4-FOR	AGCGCCTCTTTTTTTGCTGTGGATACCGATATGCGTGT TTATTCCGGGGATCCGTCGACC
yohI-P1-REV	CCCGGCACACGTTCTGGACGCTGTAGAGATTAGATTT TAGTGTAGGCTGGAGCTGCTTC
yohI-E1	TGTGGAAAGGGAAATCGC
yohI-E2	GCAGCGATAGTATCTG
acrA-E1	ACA TCC AGG ATG TGT TGT CG
acrA-E2	CAC TGT TGG AGG ACA TAT AC
acrA-P1-FOR	GAC CAA TTT GAA ATC GGA CAC TCG AGG TTT ACA TAT GAA CTG TAG GCT GGA GCT GCT TCG
acrA-P2-REV	AGA AAT TAG GCA TGT CTT AAC GGC TCC TGT TTA AGT TAA GCA TAT GAA TAT CCT CCT TAG
acrB-E2	GAC AGG AGA AAA TAG CCA GG
acrB-E1	GTT GGC GCT GAT AAC AAA GTG
acrB-P1-FOR	GTC TTA ACT TAA ACA GGA GCC GTT AAG ACA TGC CTA ATT TCT GTA GGC TGG AGC TGC TTC G
acrB-P2-REV	CTT GCG CGG CCT TAT CAA CAG TGA GCA AAT CAG CGA TGT TCC ATA TGA ATA TCC TCC TTA G
acrD-E1	CCAACAAGGAAGAGAGTCAG
acrD-E2	TTGAACGTGAACTGGGGAAC
acrF-P4-FOR	CTGCAGCGAAAACGGCGCAATAAGGTAACCGTACATGGCAATTCCGG GGATCCGTCGACC
acrF-P1-REV	AGGCGTCCGAAGACGCCTCTGTTTACCGGTTAATCATGATTGTAGGCT GGAGCTGCTTCG
acrF-E1	GCTTCCCGGTATGTTTGTTC
acrF-E2	GTATTGGTATGAACCAGCCG
emrA-E1	CTG GTC ATC ACT TAG CAC AG
emrA-E2	GAC TTC GCC AAA ACG TTT CG
emrA-P1-FOR	CAG CCG ATG TGA ATA ATA AGA TCG TGG AGA ACA ACA TGA GTG TAG GCT GGA GCT GCT TCG
emrA-P2-REV	GCG GTT TTT GCT GTT GCA TCA CAC GCA CCT CAG GAT TAA CCA TAT GAA TAT CCT CCT TAG
macA-E1	GTA ATC TGA AGT GTA CCA GG
macA-E2	GAC AAC AGA TGG TAG CGC TG
macA-P1-FOR	TTA CTT TCT GGT TAA AAT TTT GCC GTC AGG GTT TCT ATG CTG TAG GCT GGA GCT GCT TCG
macA-P2-REV	TAC GAC TCA CAT TGC ACA GTT CAA GCA ATG CCG TCA TGG CCA TAT GAA TAT CCT CCT TAG
mdfA-E1	GTA ACA GGG AGG AAA AGC AG
mdfA-E2	CGG CAA CGA TAT TGA GAT GC

mdfA-P1-FOR	CAC TCT GCG CGG TTT TTA TTG GCG AAG AGA TGG TAT GCA GTG TAG GCT GGA GCT GCT TCG
mdfA-P2-REV	GAT AAC AGA AAG CTT ATC AGG CGT AGC CGC TCT CTC AGA CCA TAT GAA TAT CCT CCT TAG
mdsA-E1	TAT TCA GGC TGC ACG GTT C
mdsA-E2	GAC CAT CAA TGG CCA TCT G
mdsA-P1-FOR	GGA AAC GAC ATG CGT AGA ACA TTC AAA ATT ATG TTG ATA GTG TAG GCT GGA GCT GCT TCG
mdsA-P2-REV	GAT GGG GCG TGC AAT GAA AAA GTG GGT GAA TTT CAT TGG CGC CAT ATG AAT ATC CTC CTT AG
mdtA-E1	GAA ATC GGT GGC TTA CCT TC
mdtA-E2	GAC ATC TGC TTC AGT CCT GA
mdtA-P1-FOR	CGC AAA ACG TTT CAG GAT GAG AAA CTT ATA CCG ATG AAA GTG TAG GCT GGA GCT GCT TCG
mdtA-P2-REV	GGC CGC CCG TGC TGC CCG GAG GTA ATA CCT GCA TCA GGC GCA TAT GAA TAT CCT CCT TAG
mdtK-E1	CCAGCATGTAGTCAGGTAAC
mdtK-E2	TATGCTTCTTCAGGCCACTG
mdtK-P1-FOR	TACGTGCAGAAGTATACCAGTGAAGCGCGTCAGTTATTAGTGTAGGC TGGAGCTGCTTCG
mdtK-P2-REV	CCATGCGACGGCTATCGTCGTATTATCGCGCAGCGCGCTGCATATGA ATATCCTCCTTAG
RT-acrA-FOR	TTTGCGCGCCATCTTCCC
RT-acrA-REV	GACGTGCGCGAACGAAC
acrB-RT-PCR FOR	CGCTGGGCGTATCTATTAGC
acrB-RT-PCR REV	CGCGATCGATAAAGTCGTTT
RT-acrD-FOR	GCTGCACAAAGGCGAACA
RT-acrD-REV	TACGATTGAAGGTACGGTTAAACCA
RT-acrF-FOR	TGTCGCCTCTAACATTAAGGATTCT
RT-acrF-REV	CGTACTGTGCGCCAAATAGCT
RT-emrA-FOR	TCGGTCTGGATATGGGAACAG
RT-emrA-REV	AGTTACCCGTCGCATTTTGC
RT-macA-FOR	GGTATCGCAACAGGATCTGGAT
RT-macA-REV	GCCAATACGCGCCTGTTTA
RT-mdfA-FOR	CCGCGATTCAGGAGTCCTT
RT-mdfA-REV	CGCGATTAACGCCACGTT
RT-mdsA-FOR	CCGTGCTGATTGACGATCT
RT-mdsA-REV	CTCCACCTGATTCTCCTTGC

RT-mdtA-FOR	GGTGTGTTGCTCAGTCTGGA
RT-mdtA-REV	AGCGTATCGTCCTGATTGGT
RT-mdtK-FOR	GAGGTCAGGCCGATGATGAA
RT-mdtK-REV	ATTTGGTGGTGGATCGTATGG
RT-hdfR-FOR	TTGGCGTTATATTGCAGCAG
RT-hdfR-REV	CCAGCCGCAGATAATTGAGT
ST-PCR1	GCCTTCTTATTCGGCCTTGAATTGATCATATGCGG
ST-PCR2-GATAT	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
ST-PCR2-ACGCC	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
ST-PCR3	CTTTTTCCGTGATGGTAACC
ST-PCR4	GGCCACGCGTCGACTAGTAC

Polymerase chain reaction (PCR)

For PCR reactions, a Perkin Elmer Gene-Amp PCR system 2400 thermocycler (Perkin Elmer Cetus, Waltham, Massachusetts, USA) was used. PCR reactions were carried out with 1 ng of DNA, 100 µM of dNTPs (final concentration each), 1 µM of oligonucleotides, 1mM of $MgCl_2$, and 1 U of Taq polymerase per reaction in a final volume of 100 µl. The polymerase used in these reactions was Taq Expand™ High Fidelity PCR System supplied by Roche Diagnostics GmbH.

To confirm clones and mutations, colony PCR was performed using Go Taq® Flexi DNA Polymerase (Promega Co.). In these cases, a mixture with 100 µM of dNTPs each one, 0.2 µM of oligonucleotides, 1 mM of $MgCl_2$ as final concentration and 1 U of Taq polymerase per reaction in a final volume of 25 µl was prepared. A colony was re-suspended in this mixture and was used as DNA template.

Before using any PCR product obtained for further molecular techniques; the enzyme, oligonucleotides and dNPTs were removed using the commercial Wizard® SV Gel and PCR Clean-Up System, which was supplied by Promega Co.

Semi-random, two-step PCR (ST-PCR)

The ST-PCR protocol was used to amplify genomic regions adjacent to the T-POP3 (Tet^R) insertion (Figure M1). In a first step, a PCR reaction was carried out over the selected candidates using oligonucleotides ST-PCR-1 and ST-PCR2-GATAT or ST-PCR2-ACGCC (Table M4) in a final volume of 25 µl, the protocol of this first amplification was as it follows: (i) first denaturation, 2 min at 94° C; (ii) 6 cycles of denaturation (94° C, 30 s), annealing (42° C, 30 s, -1° C per cycle), and extension (72° C, 3 minutes); (iii) 25 cycles of denaturing (94° C, 30 s), annealing (65° C, 30 s), and extension (72° C, 3 minutes); and (iv) a final incubation at 72° C for 7 min for a final extension. The second reaction was carried out with the oligonucleotides ST-PCR4 and ST-PCR3. The DNA product of the previous PCR was diluted 1:5 and 1 µl was used as DNA template. The protocol for the second PCR was: (i) first denaturation, 30 s at 94° C; (ii) 30 cycles of denaturation (94° C, 30 s), annealing (56° C, 30 s), and extension (72° C, 2 min); and (iii) a final extension at 72° C for 7 minutes to finish the extension. Final products were sequenced using the oligonucleotides ST-PCR4 and ST-PCR3.

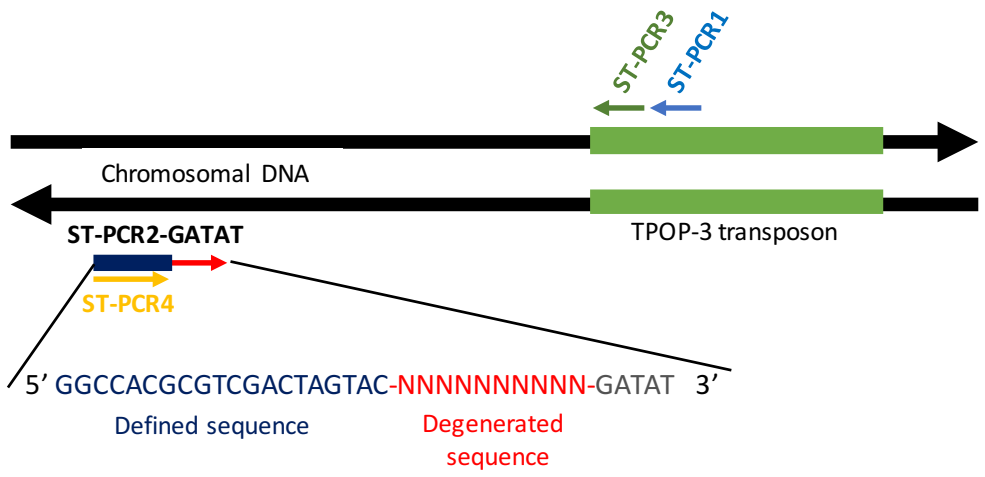

Figure M1. Representative scheme of the PCR reactions. The last five nucleotides of ST-PCR2-GATAT allows the hybridization of the oligonucleotides along the genome of *Salmonella* because they occur every certain number of base. Two kinds of ST-PCR2 oligos were used: the one finished in GATAT and another one that finished in ACGCC. Because the Salmonella genome has a G+C content of 52%, oligonucleotides ST-PCR2-GATAT will bind every 1159 bp [1/(0,26 x 0,24 x 0,24 x 0,24 x 0,24)], and oligonucleotides ST-PCR2-ACGCC will bind every 912 bp [1/(0,24 x 0,26 x 0,26 x 0,26 x 0,26)]. Figure adapted from Chun et al. (1997)[98].

Chromosomal gene disruption using PCR products

To obtain knockout mutants of chromosomal genes, the Datsenko and Wanner method was used [92]. This method is based in the λ Red recombination system. One of the reasons why *E. coli* and *Salmonella* are not transformable with linear DNA is due to the presence of intracellular exonucleases that degrade it. The λ Red system harbors *α, β* and *exo* genes that codify for the proteins Gam, Bet and Exo, respectively. Gam inhibits host exonuclease V, allowing the Bet and Exo proteins to carry out recombination of the DNA. The strategy consists in replacing the chromosomal sequence (for example gene B in figure M2) by an antibiotic resistance marker that is generated by PCR using oligonucleotides that harbor 40 nucleotides of homology with the sequence to be replaced (H1 and H2 in figure M2). λ Red recombination gene expression is carried out under an inducible promoter inside a thermosensitive low copy number plasmid (pKD46). After selection, gene resistant marker can be removed using a different plasmid (pCP20) that harbors the FLP recombinase of the 2 μ plasmid from *Saccharomyces cerevisiae*. FLP system acts over FRT repetitions ("FLP recognition target") that flank the sequence (figure M2). Plasmids that harbors Red and FLP system are thermosensitive and can be cured easily by growing the cells at 37° C.

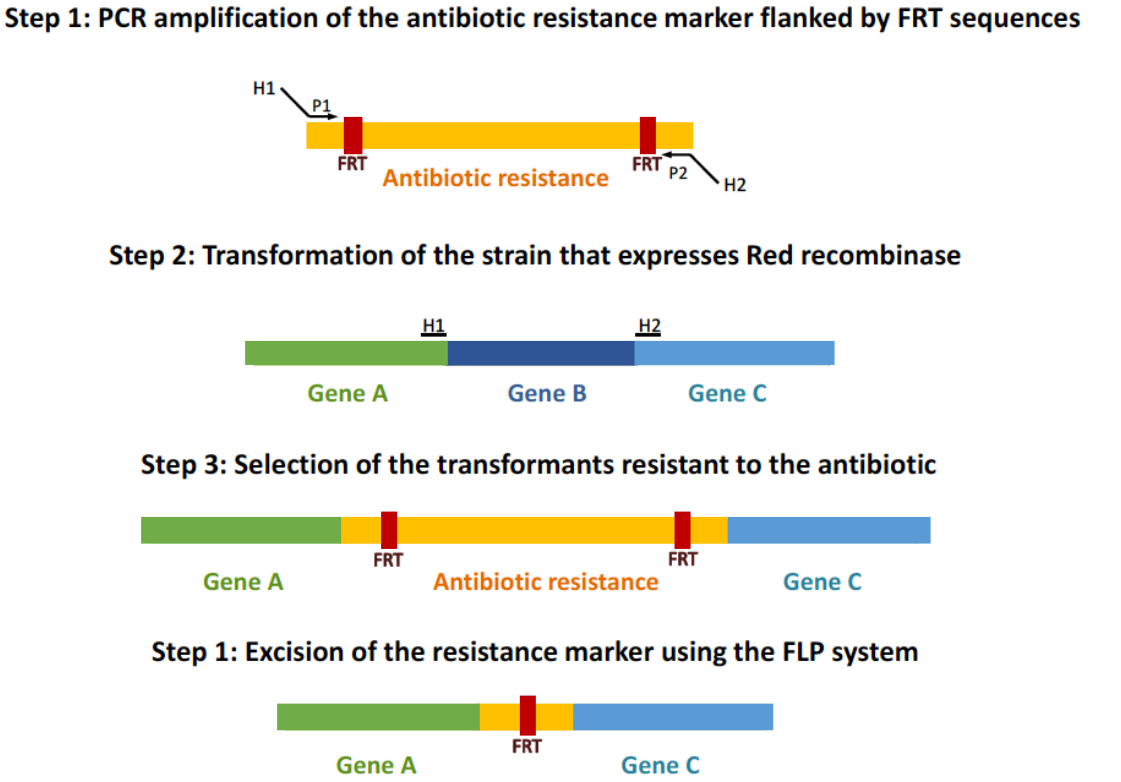

Figure M2. Scheme of the inactivation system by PCR. H1 and H2 are related to the homology regions with the disrupting sequence. P1 and P2 are the homology sequences that flank the antibiotic gene sequence. Figure adapted from Datsenko and Wanner (2000) [92].

Preparation of DNA for substitution

The plasmids used as templates in PCR reactions were pKD3 (Cm^R), pKD4 (Km^R) and pKD13 (Km^R). The oligonucleotides used had 40 nucleotides that were homologous to the genomic DNA and 20 nucleotides that were homologous with pKD3, pKD4 and pKD13. PCR reactions were carried out at 55° C for annealing temperature and 2 minutes for the DNA fragment extension, the enzyme employed was "Taq Expand™ High Fidelity PCR System", supplied by Roche Diagnostics GmbH. The PCR product obtained was subjected to an agarose gel electrophoresis and the amplified band was purified using the commercial system "Wizard® SV Gel and PCR Clean-Up System", which was supplied by Promega Co.

Cell transformation

Competent cells of the wild type strain, which harbored the pKD46 plasmid, were

prepared. This plasmid expresses λ Red system under the *araB* promoter, which is inducible by arabinose. Cultures grown in LB with ampicillin at 30° C were diluted 1:100 in LB with Ampicillin and arabinose (1 mM) and they were incubated in a shaker at 30° C until they reached a 0.5 OD_{600}. The competent cells were prepared and electroporation was done as described previously.

Excision of the resistance marker

After the substitution of the genomic genes with antibiotic resistance cassettes (Km or Cm), mutations were transferred to different genomic backgrounds by transduction with P22 and selection in the appropriate media. When it was necessary the resistance marker of the host was excised by transducing the plasmid pCP20 with the P22 phage. This transduction was incubated at 30° C for 1 h and was spread in LB with ampicillin. To eliminate the plasmid, EBU plates were prepared without antibiotic and incubated at 37° C. To confirm the excision of the marker, the strains were streaked in plates of LB ampicillin and plates of LB with chloramphenicol or kanamycin. The excision of the antibiotic marker was also observed by colony PCR with external oligonucleotides.

Strain construction by transductional transfer of genetic markers

Genetic markers were transferred from one strain to another by transduction. The recipient strain was transduced using a P22 lysate from a strain with the desirable genetic marker. All the markers used in this thesis were selected directly by spreading the transduction mixture on selective media. If necessary, acquisition of the marker by the transductant was confirmed by PCR or phenotypic analysis.

Adaptation assays in bile gradient plates

Stationary cultures in LB broth were diluted 25 times (100µL culture in 2.5mL LB) and incubated at 37°C with shaking until they reached a concentration of approximately $1.2x10^8$ cells/mL. From this exponential phase culture 100µL were plated in 0-30% ox bile agar gradient plates, a technique that has been described before for antibiotics [99], and another 100µL were diluted in 2.5 mL of LB with 5% DOC and incubated o/n at 37°C with shaking. The following day, the cultures were diluted like the day before in LB with

5% DOC and incubated until there where roughly $1.2x10^8$ cells/mL; 100µL of the culture were plated in the ox bile gradient plates while other 100 µL were cultured o/n in LB. The overnight cultures where diluted in LB like the previous days and plated again in ox bile gradient plates. All the plates were incubated at 37°C for two days, growth was visually monitored.

Determination of minimal inhibitory concentrations of sodium deoxycholate

Exponential cultures in LB broth were prepared, and samples containing around $3x10^2$ colony-forming-units (CFU) were transferred to polypropylene 96 well microtiter plates (Soria Genlab) containing known amounts of sodium deoxycholate (DOC). After 12 h incubation at 37°C, growth was visually monitored. This technique was also used to monitor DOC adaptation.

In vivo assays: Isolation of bile hyper-resistant candidates from mice gallbladders.

Eight-week-old female BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) were inoculated with the wild type strain SL1344. Bacterial cultures were previously grown overnight at 37°C in LB without shaking. Oral inoculation was performed by feeding the mice with 25 µl of NaCl 0.9% containing 0.1% lactose and 10^8 bacterial colony- forming units (c.f.u.). Bacteria were recovered from the gallbladder of infected mice 5 days after the oral infection. To select SL1344 derivatives, the isolates were plated in LB Streptomycin and grown overnight at 37°C. Also, growing the isolates in LB Str (bile-free media) was the first step to differentiate stable bile resistant mutants from adapted bacteria.

The following day, replica plating was performed to all the candidates in LB agar plates that contained increasing bile concentrations (12, 14, 16, 18 and 20% ox bile extract), the plates were then incubated overnight at 37°C. Bile resistant mutants were picked and its phenotypical stability was confirmed by DOC liquid MICs in 96 well plates. Once

the candidates were selected, gDNA was isolated as described and the samples were sent for whole genome sequencing. As a control, our laboratory wt strain genome was also sequenced.

DNA sequencing

Plasmid DNA and chromosomal DNA obtained by PCR were sequenced in the sequencing service of Stab Vida (Oeiras, Portugal).

Whole bacterial genome sequencing was done by the Biology Sequencing Service in the University of Seville, (Centros de Investigación, Tecnología e Innovación CITIUS, Edificio Celestino Mutis, Seville, Spain). As a control, the genome of the laboratory stock of *S. enterica* SL1344 was also sequenced. Briefly, draft genome sequences were obtained using Roche 454 FLX+ technology on a GS FLX titanium system (Roche Diagnostic, GmbH) [100]. Samples were prepared according to the GS Rapid Library Preparation Method Manual. Emulsion PCR and 454 pyrosequencing were performed following the manufacturer's instructions [101].

DNA sequence analysis

Bioinformatics analysis of DNA sequences was performed using the algorithms of molecular biology of the National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov.

Whole genome sequences were analyzed in collaboration with the Institute of Integrative Biology, Department of Functional and Comparative Genomics in the University of Liverpool. The Burrow-Wheeler Alignment tool (BWA) [102] was the algorithm of choice for such purpose. The first step was the alignment of the 454 GS FLX+ / XL+ sequence reads to the reference genome, in our case the *S. enterica* SL1344 genome sequence (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs), to identify DNA sequence differences. The alignment tool BWA consists of three algorithms: BWA-backtrack, BWA-SW and BWA-MEM. The first algorithm is designed for

Illumina sequence reads up to 100bp, while the rest two for longer sequences ranged from 70bp to 1Mbp [102,103]l. In our case, the BWA-MEM algorithms was employed, which is recommended for high-quality queries as it is faster and more accurate. BWA-MEM also has better performance than BWA-backtrack for 70-100bp Illumina reads (Li and Durbin, unpublished). The genome sequence of the laboratory stock of *S. enterica* SL1344 was also analyzed and as a control.

RNA manipulation

RNA extraction based in TRIsure™ method

To prepare cells for RNA extraction, 5 ml of fresh LB ± 5% DOC was inoculated with 100µL from an overnight bacterial culture, and incubated with shaking at 37°C. Three biological replicates were performed for each strain. For exponential phase cultures ($O.D._{600} \approx 0.6$), a 2 ml aliquote from each culture was saved 1.5 and 3h later, depending if the culture was in LB or in LB + 5% DOC, respectively; for stationary phase cultures ($O.D._{600} \approx 2$), a 2 mL aliquote of o/n cultures was saved (>12h). The samples were centrifuged at 13000rpm, 4° C for 2 minutes (Centrifuge 5415 R, Eppendorf). The pellet was washed three times with NaCl 0.9% and resuspended in 100 µl of a solution of lysozyme (3mg/ml; Sigma Chemical Co.). Cell lysis was facilitated by a freeze-thaw cycle. After lysis, RNA was extracted using 1 ml of TRIsure reagent (Bioline, Taunton, Massachusetts, USA) was added, and the preparation was incubated for 5 min at room temperature. Samples were centrifuged at 4° C at 13000 rpm for 10 min. The supernatants were recovered and poured out in clean tubes (eliminating the genomic DNA in this step). 200 µl of chloroform were added, and the samples were vortexed for 15 s and centrifuged for 15 min at 4° C and 13000 rpm. The supernatants were carefully recovered, avoiding recovering the interphase, and transferred to clean tubes. 500 µl of isopropanol were added. Samples were mixed by inversion 2-3 times and they were incubated at room temperature for 10 min, followed by a 10 min centrifugation. The supernatants were discarded and the pellets were washed with cold 70% ethanol. The samples were centrifuged at 4° C and 13000 rpm for 5 min. The supernatants were discarded and pellets were air-dried and then kept at -20° C until use. The pellets were re-suspended

in 30 µl of RNAse-free H_2O (autoclaved ddH_2O is enough). To obtain a homogeneous mixture, the samples were incubated at 65° C for a few minutes. The quantity and quality of the RNA was determined using a ND-1000 spectrophotometer (NanoDrop Technologies).

RNA phenol extraction

Whenever RNA samples were not clean enough, they were subjected to phenol treatment. First, ddH_2O was added until a final volume of 150 µl. The same volume of phenol was then added and the preparation was mixed by vortexing. The samples were centrifuged at 4° C and 13000 rpm for 5 min. The aqueous layer was recovered in a clean tube. The same volume of chloroform: isoamyl acid (24:1) was then added. The samples were mixed by vortexing and centrifuged at 4° C at 13000 rpm for 5 min. The aqueous layer was recovered and 2.5 volumes of ethanol and 1:10 volumes of sodium acetate 3 M pH 5.2 were added. The samples were precipitated at -20° C for at least 30 min. After precipitation, the samples were centrifuged at 4° C and 13000 rpm for 30 min, and washed with ethanol 70 %.

Quantitative RT-PCR (qRT-PCR)

Retrotranscription was performed using QuantiTect® Reverse Transcription Kit (Qiagen, Venlo, The Netherlands) following manufacturer's instructions. Quantitative RT-PCR reactions were performed in a LightCycler 480 II apparatus (Roche). Each reaction was carried out in a total volume of 10 µl on a 480-well optical reaction plate (Roche) containing 5 µl SYBR, 0.5 µl DYE II (Takara, Japan), 4.6 µl cDNA (1/10 dilution) and two gene- specific primers at a final concentration of 0.2 mM. Real-time cycling conditions were as follows: (i) 95°C for 10 min and (ii) 40 cycles at 95°C for 15s, 60°C for 1min. A non RT control was included for each primer set. Triplicates were run for each reaction, and the Ct value is averaged from them. The expression of the target genes was normalized to the expression of a constitutive gene used as internal control. Absence of primer dimers was corroborated by running a dissociation curve at the end of each experiment to determine the melting temperature of the amplicon. Melting curve analysis verified that each reaction contained a single PCR product. Gene-specific

primers were designed with Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and are listed in Table M4.

Quantification of qRT-PCR results

For quantification, the efficiency of each primer pair was determined to be between 90%-110%, following the instructions for efficiency determination described in the "Guide to Performing Relative Quantification of Gene Expression Using Real-Time Quantitative PCR" (Applied Biosystems). These efficiencies indicate that the amount of DNA is doubled in each PCR cycle, and allows for direct comparison between different genes. Relative RNA levels were determined using the ΔΔCt method as described in the above mentioned guide. Briefly, each gene *Ct* value is normalized to the *Ct* value for the internal control (*hdfR*), which gives the ΔCt value. This value is then related to a given gene in the reference strain (*SL1344*, in this case) giving us the ΔΔCt value. Since the amount of DNA doubles in each PCR cycle, the relative amount of input cDNA can be determined by using the formula $2^{-\Delta\Delta Ct}$. Each ΔΔCt determination was performed at least in three different RNA samples (three biological replicates), and the results presented here are a representative example of such determinations.

Flow cytometry

Flow cytometry was used to monitor the efflux pump AcrAB activity over time. Data acquisition and analysis were performed using a Cytomics FC500-MPL cytometer (Beckman Coulter). Data were collected for 100000 events per sample, and were analyzed with CXP and FlowJo 8.7 Software.

To study AcrAB activity, accumulation and efflux of EtBr was monitored on a real-time basis [104,105].. EtBr was excited at 488 nm, and fluorescence was detected using a 585-nm filter. To study AcrAB activity with DOC, exponential cultures of strains SV7636 (*ΔacrDmdtK mdfA emrA acrF mdtA, macA::Km, mdsA::Cm*) and SL1344 were diluted with and without 5% DOC and incubated for 2 hours (for exponential phase) and o/n (for stationary phase). EtBr was then added (1 μg/mL) and the cultures were incubated for an additional 60 minutes at 37°C in the dark to permit EtBr accumulation. Finally, 0.5 mL

of each culture was taken, the bacterial suspension was centrifuged for 3 min at 13000 rpm and the pellet was resuspended in PBS for EtBr fluorescence measurement. To study AcrAB activity during adaptation to DOC, the same strains were cultured in 20 mL LB up to an OD≈0.35, when DOC was added to a final concentration of 5%. 1mL samples were taken every hour, treated with EtBr as described previously, and analyzed in the cytometer for 8 hours.

Statistical analysis

To calculate averages and standard deviations of the experiments performed, “Microsoft Excel” was employed. In addition, the Student’s t test was used to determine if the differences in our experiments were statistically significant.

RESULTS

CHAPTER 1: EXPLORATION OF CELLULAR FUNCTIONS ESSENTIAL FOR BILE ADAPTATION AND RESISTANCE IN *Salmonella enterica*

Surveys of bile adaptation in bile-sensitive mutants

The goal of this assay was to ascertain if different bile-sensitive mutants were able to adapt or not to survive bile after previous incubation with sub-lethal concentrations of sodium deoxycholate (DOC). The aim was to try to find if the adaptation process was a synergic process depending on several gene functions or if it was mostly dependent on a specific one. The mutants used carried loss-of-function mutations in the following genes:

- *dam*, encoding a DNA adenine methylase involved in genome defense, DNA replication and repair, nucleoid segregation, regulation of gene expression, control of transposition, and host-pathogen interactions [106–108]. A *dam* mutation causes pleiotropic defects but is not lethal [109].
- *damX,* a gene whose protein localizes in the septal ring in *E.coli*. Loss of DamX results in a variety of division phenotypes, suggesting a role in cytokinesis [110]. In *Salmonella enterica*, DamX is located in the inner membrane and its loss causes severe sensitivity to bile [111].
- *phoP*, a gene that encodes one of the components of the PhoPQ sensor system, which comprises a membrane bound sensor kinase PhoQ (which has a binding domain and a kinase/phosphatase domain) and a response regulator PhoP in the bacterial cytoplasm [112]. PhoPQ is essential for the virulence of Gram-negative bacterial pathogens in humans and mice [113]. According to van Velkingburg *et. al.* (1999), the PhoPQ system is required for enhanced resistance to bile in *Salmonella* [86].
- *rpoS,* the gene encoding sigma factor RpoS, which is required for expression of genes involved in a variety of environmental stresses (starvation, oxidation, low pH) [114]. In addition, RpoS is required for *Salmonella* virulence in mice [115]. This gene is up-regulated by 5% sodium deoxycholate and *rpoS* mutants are bile-sensitive [88].
- *wecD*, a gene involved in the synthesis of enterobacterial common antigen (ECA). ECA plays a role in *Salmonella* virulence, particularly upon oral infection, and may protect the pathogen from bile salts [73].

- *zapB*, a gene that codes for a non essential cell division factor in Z-ring assembly [116]. *Salmonella enterica zapB* mutants are bile sensitive (Hernandez, 2012, unpublished data).
- *tolC,* which codes for the outer membrane channel of the AcrAB efflux pump, and plays a role in the excretion of a wide range of molecules, including antibiotics [117].
- *acrA,* a gene encoding another component of the AcraB efflux pump. AcrA is a periplasmic lipoprotein that may perform membrane fusion by bridging the outer and inner membranes [77].
- *acrB*, which codes for an integral membrane protein with 12 membrane spanning α-helices, located in the cytoplasmic membrane [78]. Thes *arcA, acrB*, and *tolC* genes form part of the tripartite efflux pump system AcrAB-TolC, which has been characterized in detail in *E.coli* [80,83] and exists also in *S. enterica* [79,81,118].

The adaptation concentrations were determined by overnight incubation of exponential phase cultures of these strains with increasing concentrations of sodium deoxycholate (DOC) and monitoring their growth to define the sub-lethal levels of this compound for each strain (maximal concentration that permitted survival). The concentrations were 0.5, 1, 2, 3, 4 and 5% DOC. The maximal concentrations that permitted survival of each strain are shown on table C1.1. The *tolC*, *acrA* and *acrB* strains, marked with an asterisk (*), lack survival concentration values because they were unable to grow even at low concentrations of DOC (0.5% and 1%).

Table C1.1. Maximal concentrations of DOC (%) that permitted survival of bile sensitive strains.

Strain	Maximal survival concentration (% DOC)
SL 1344 (WT)	5%
Δdam	1%
damX::Km	4%
phoP::Tn10	5%
rpoS::Ap	5%
wecD::Km	4%
zapB::Km	5%
*tolC::Km**	-
*acrA::Km**	-
*acrB::Km**	-

The following day, aliquots of these cultures where plated on ox bile extract gradient plates (0-30%) and incubated overnight at 37° C. As controls, cultures of the same strains grown oin LB (in other words, not pre-exposed to bile nor to sodium deoxycholate) were also plated on bile gradient plates. An additional control was strain BR6, a mutant hiper-resistant to bile carrying a mutant allele of *lptC (yrbK)* (Hernandez et al. 2012). Most of the bile sensitive mutants under study were able to adapt, increasing their level of bile resistance when compared to their resistance levels in LB (Figure C1.1). In some cases, including the strains used as controls (wild type SL1344 and the bile resistant mutant BR6), a bacterial lawn appeared, which implied that the resistance level could be over 30% bile. The only mutants that could not adapt to bile and showed a very high susceptibility to DOC where *acrA::*Km, *acrB::*Km and *tolC::*Km.

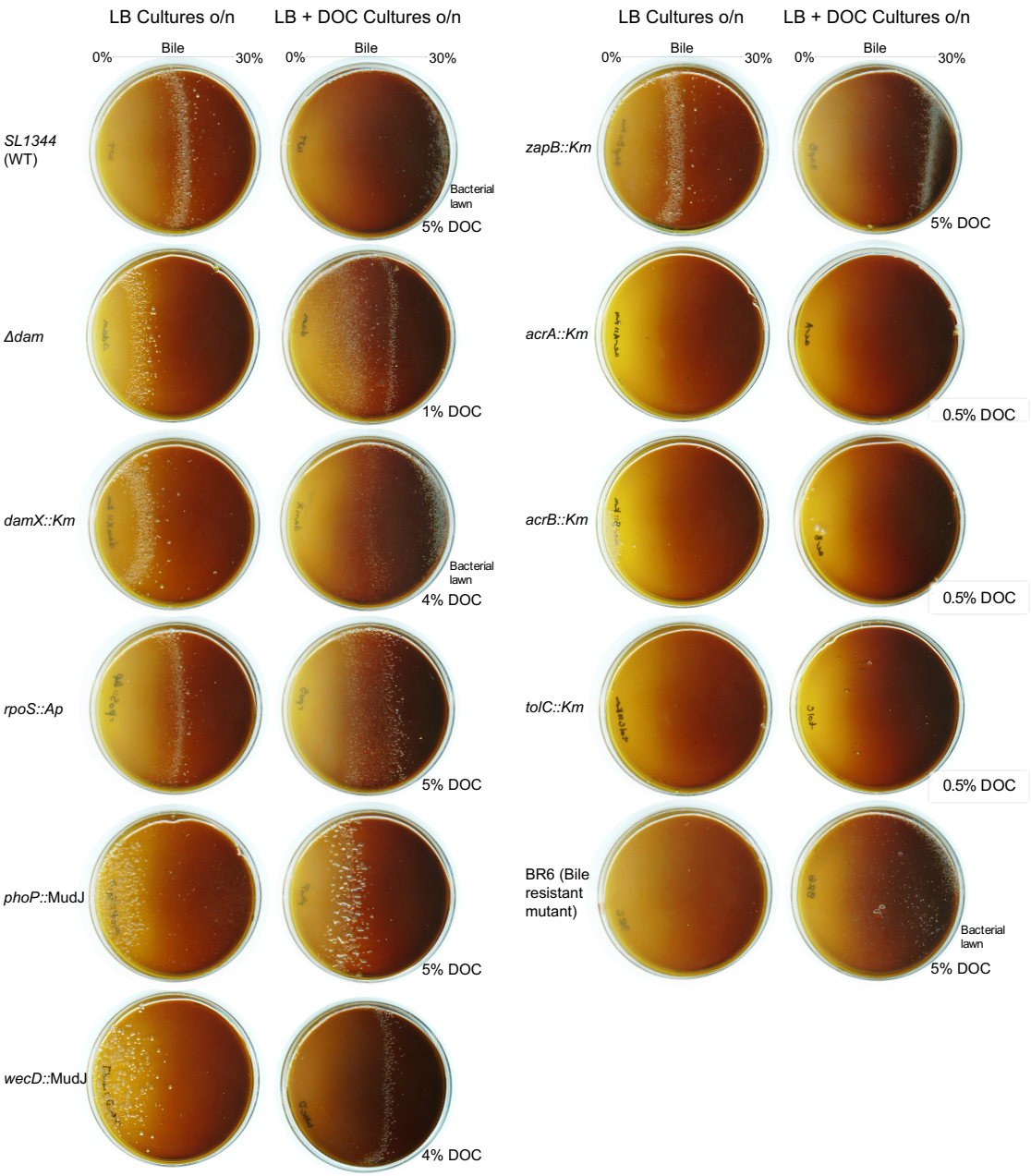

Figure C1.1. Adaptation assays in 0-30% ox bile gradient plates of bile sensitive mutants. Plates on the left side of each column show the bile resistance levels of each strain cultured overnight in LB broth. On the right side of each column the plates of the same strains cultured overnight in LB with their sub-lethal concentrations of sodium deoxycholate (DOC). In the wt (SL1344), *damX*::Km and BR6 (bile resistant mutant) strains, a bacterial lawn appeared (BR6 exhibits a bacterial lawn before and after DOC exposure). The percentage of DOC used for adaptation is in the right side of the adapted strain plates.

Suppression of *acrA*, *acrB* and/or *tolC* bile sensitive phenotypes: genetic screen with a pBR328 plasmid genomic library.

A *S. enterica* genomic library constructed on the pBR328 plasmid was used to search for bile-sensitive phenotype suppressors in the *acrA::*Km background (strain SV7248). The pBR328-based multicopy plasmid library was transduced using P22 HT phage, and transductants were selected in LB agar + Cm + 0.5% DOC. Since the *acrA* mutation confers high sensitivity to bile and/or bile salts, the ability of the transductants to grow on sodium deoxycholate indicated an increased level of bile resistance. The next day the colonies obtained were replicated to LB + Cm agar plates containing increasing sodium deoxycholate (DOC) concentrations (from 1% to 7%).

Nine independent pBR328 genomic library pools (from the laboratory collection) were used to transduce the *acrA* mutant and the wild type. Ten transductions per pool were performed, thus reaching a total of 90. The number of transductants differed from one pool to another. As a control to determine the efficiency of the plasmid library, 5 transductions with 5 independent pools were performed in the wild type strain (SL1344). Table C1.2 shows the number of transductants obtained

Table C1.2. Number of transductant colonies in wild type (SL1344) and *acrA* backgrounds grown in LB + Cm with 0.5% sodium deoxycholate agar plates.

	Pools								
Strain	1	2	3	4	5	6	7	8	9
acrA::Km	8	19	26	19	31	27	31	25	15
SL1344 (wt)	126	246	421	-	-	346	-	-	408

In a wild type background, a higher number of transductant colonies appeared, a result coherent with the higher resistance level of this strain compared with the *acrA* mutant.

Each of the transductant colonies obtained in the *acrA* background, now carrying a pBR328 derivative, was replicated to LB agar plates containing increasing concentrations of sodium deoxycholate (from 1 to 10%). The agar also contained Cm to maintain the plasmid. Out of the 210 independent colonies obtained, 10 were able to grow up to 7% DOC. This meant that the unknown fragments in the library contained one or several

genes whose expression from a multicopy plasmid somewhat restored the levels of bile resistance in the *acrA* background. As an additional control, an empty pBR328 vector was transformed into the *acrA*::Km strain, and was replica-plated to the LB agar plates with increasing concentrations of sodium deoxycholate along with the candidates. This strain showed a bile sensitivity phenotype like the *acrA* mutant, thereby confirming that suppression of bile sensitivity was due to the inserts and not to the plasmid backbone.

The 10 candidates with increased DOC resistance were cultured in LB broth + Cm. After overnight growth, the pBR328 derivatives were isolated and sequenced using specific primers flanking the insertion site (table M4). DNA sequencing revealed that most pBR328 library plasmids carried fragments that contained efflux pump genes, specifically *acrF*, *acrE* and *acrB*. Other frequent hits were the genes *yhdJ*, a non essential adenine DNA methyltransferase [119] and *yhdU,* a protein with unkown function annotated as a putative preplasmic protein in the http://www.uniprot.org website.

Loci with only one occurrence included:

- *aefA,* which codes for an integral membrane protein and could be involved in transmembrane transport (http://www.uniprot.org).
- *envR*, a TetR-family regulator. These regulators are, in general, involved in adaptive responses against environmental changes [120]. However no specific function for the *envR* gene has been described.
- *polB*, which codes for the DNA polymerase II enzyme. This enzyme plays a role in stress induced, RpoS-dependent mutagenesis, a response activated under adverse conditions such as starvation or antibiotic stress [121].
- *hsdR*, a type-III restriction enzyme with no known function in bile resistance or other stress responses.
- Finally, four genes with products of unknown function were also identified.

Detailed information about the findings on this screen are in table C1.3.

Table C1.3. Identification of genomic inserts from pBR328 derivatives obtained from the increased sodium deoxycholate resistance candidates found on the screen. For each candidate the genomic region of the fragments (position in the chromosome) and the genes present in such region are indicated.

Candidate	Genomic region inserted	Genes within the region
1.17	529239-535286	acriflavin resistance protein B (*acrB*) integral membrane protein AefA (*aefA*)
1.45	3578680-3585394	*acrF* CcrM-like DNA adenine methyltransferase (*yhdJ*) hypothetical exported protein (*yhdU*)
2.33	3578680-3585394	*acrF* *yhdJ* *yhdU*
2.45	3581176-3584420 531164-531876	acriflavine resistance protein E (protein envc) (*acrE*) *acrF* acriflavin resistance protein B (*acrB*) hypothetical membrane protein (SL1344_3361) TetR-family transcriptional regulator (*envR*)
3.21	3578680-3585394	*acrF* *yhdJ* *yhdU*
3.33	3578680-3585394	*acrF* *yhdJ* *yhdU*
4.16	3578680-3585394	*acrF* *yhdJ* *yhdU*
4.18	113899-115672	DNA polymerase II (*polB)* probable secreted protein (SL1344_0099)
4.23	4796467-4803576	hypothetical protein (SL1344_4451) conserved hypothetical protein (SL1344_4451) type III restriction enzyme (*hsdR*)
4.24	3578680-3585394	*acrF* *yhdJ* *yhdU*

The results from this genetic screen were interesting enough to focus the following stages of our research project into the description of the roles that efflux systems might have in the process of adaptation and resistance to bile in *Salmonella enterica*.

T-POP transposon genetic screen: looking for bile resistant phenotypes.

A genetic screen based on the T-POP3 transposon [95] was used to search for genes that could alter bile resistance levels either by mutation (gene disruption) or by increased expression above normal levels. For this purpose, the wild type strain (SL1344) was subjected to mutagenesis using a TPOP3 library from the laboratory collection. Mutagenesis involved transduction with P22 HT phage. Isolates carrying T-POP3 insertions were selected on LB agat + Tc + 7% sodium deoxycholate.

One hundred and twenty colonies were obtained; these were individually analyzed by determination of minimal inhibitory concentrations of sodium deoxycholate (DOC) in 96-well plates. The MICs were done in two conditions, with and without tetracycline. This is done because gene disruption due to T-POP3 insertion is insensitive to the presence of tetracycline, while increased expression of the genome regions surrounding T-POP3 is tetracycline-dependent [95]. Tetracycline induces the expression of the outward pointing *tetRA* promoters present in the transposon, thus allowing the transcription of adjacent genes [95,96].

The sodium deoxycholate MICs with and without Tc revealed that, out of the 120 candidates, 10 showed a higher DOC resistance level. However, none of the candidates showed enhanced DOC resistance level in presence of Tc. This indicates that the candidates found presented a higher DOC resistance level due to gene inactivation rather than to increased gene expression.

To identify the regions where the T-POP3 element had inserted, the semi-random two-step polymerase chain reaction (ST-PCR) method was employed. The list of genes identified are shown in table C1.4.

Table C1.4. Identification of the T-POP3 transposon insertion regions on the different bile resistant candidates found on the screen. For each candidate the genomic region of the transposon (position in the chromosome) and the genes present in such region are denoted.

Candidate	T-POP3 insertion position	Gene
A6-4A	135302	LysR-type transcription regulator (*leuO*)
B6-16A	2455187	hypothetical membrane protein SL1344_2311 (*ulaA*)
4.7	2156785	UDP-glucose 6-dehydrogenase (*udg*)
11.7B	2060185	bacteriophage terminase (large subunit)
6.1.1	3045128	Type III secretion system effector protein (*sptP*)
6.2.1	2268871	conserved hypothetical protein (*yohI*)
8.1.1A	2060845	predicted bacteriophage protein
8.2.4	2373343	hypothetical MR-MLE-family protein (SL1344_2242)
8.2.6A	2440118	hypothetical aminotransferase (*yfbQ*)
8.2.7A	388445	hypothetical rtn protein (SL1344_0338)

Since the phenotypes observed were due to gene inactivation rather than increased expression of genes flanking the T-POP3 insertion site, deletion mutants were constructed with the lambda Red gene disruption method [92]. Two candidates (8.1.1A and 11.7B) were left out as their genes did not belong to the *Salmonella enterica* genome but they were bacteriophage protein genes.

Determination of the minimal inhibitory concentration of sodium deoxycholate for each of the mutants showed no differential bile resistance level when compared with the wild type strain (SL1344) (Figure C1.2).

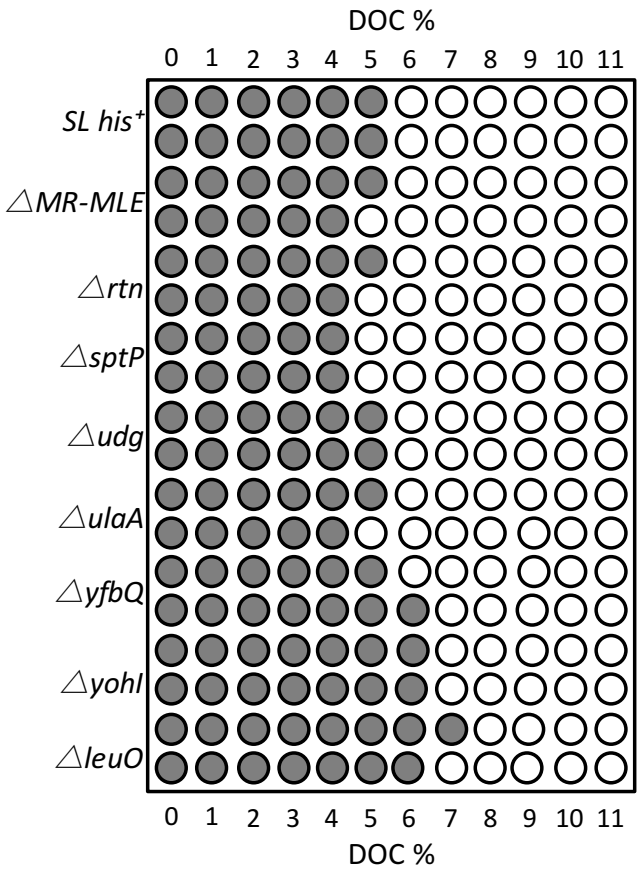

Figure C1.2. Minimal inhibitory concentration (MICs) of sodium deoxycholate (DOC) for *Salmonella enterica* strains mutants for the genes identifies in the T-POP3 genetic screen. MIC values were compared with the wt strain (SL1344 *his*$^{+}$)..

The results on this screen did not provide interesting nor conclusive results on the role these genes could play in bile resistance or adaptation in *Salmonella*. Therefore, the results of this screen were not further analyzed.

CHAPTER 2: ESSENTIAL ROLE OF AcrAB-MEDIATED EFLLUX IN *Salmonella enterica* ADAPTATION TO BILE

Surveys of adaptation to bile in mutants lacking efflux systems

The results obtained for adaptation in bile sensitive mutants showed that only efflux pumps related mutants (*acrA*, *acrB* and *tolC*) were not able to adapt and had a very high susceptibility to bile. These lead us to study the role in bile resistance and adaptation of the other eight efflux systems reported in *S.enterica* [75,81]. Therefore, single efflux pump mutants were constructed (Table M1), and minimal inhibitory concentrations (MICs) of sodium deoxycholate (DOC) were determined. The results confirmed, that not only AcrAB is essential for bile resistance [122], but also that the other efflux pumps have a minimal contribution in such resistance, having MICs of DOC values very similar to that of the wt strain (Figure C2.1). These mutants were able to adapt when cultured overnight with 5% DOC.

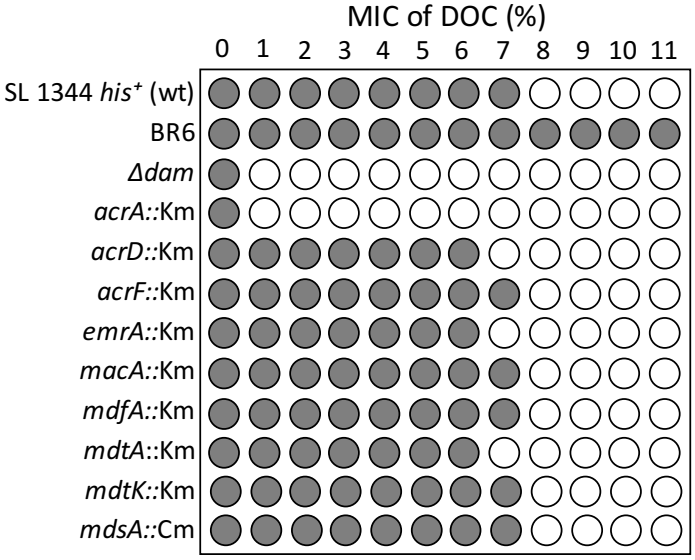

Figure C2.1. Minimal inhibitory concentration (MICs) of sodium deoxycholate (DOC) for *Salmonella enterica* strains lacking efflux systems. MIC values were compared with the wt strain (SL1344 *his*$^{+}$), a bile resistant mutant (BR6, a previously described *yrbK* mutant [88]) and a bile sensitive strain (*Δdam,* Dam methylase mutant).

The preliminary results on single efflux pump mutants led us to hypothesize that, even though bile resistance in *Salmonella* was achieved mainly by the active efflux of the AcrAB system, it could be possible that the other efflux pumps played an additional secondary role in bile resistance. To study this possibility, mutants that lacked from two to eight efflux systems were designed and constructed by sequential replacement of

each efflux pump gene with an antibiotic resistance cassete [92]. As a result, eight new mutants were obtained (Table M1): all of them having the *acrAB* genes untouched. These new strains were assayed by determining its minimal inhibitory concentration (MICs) in ox bile gradient plates (0-30%) to study their resistance level and if they were able to adapt as well (Figure C2.2).

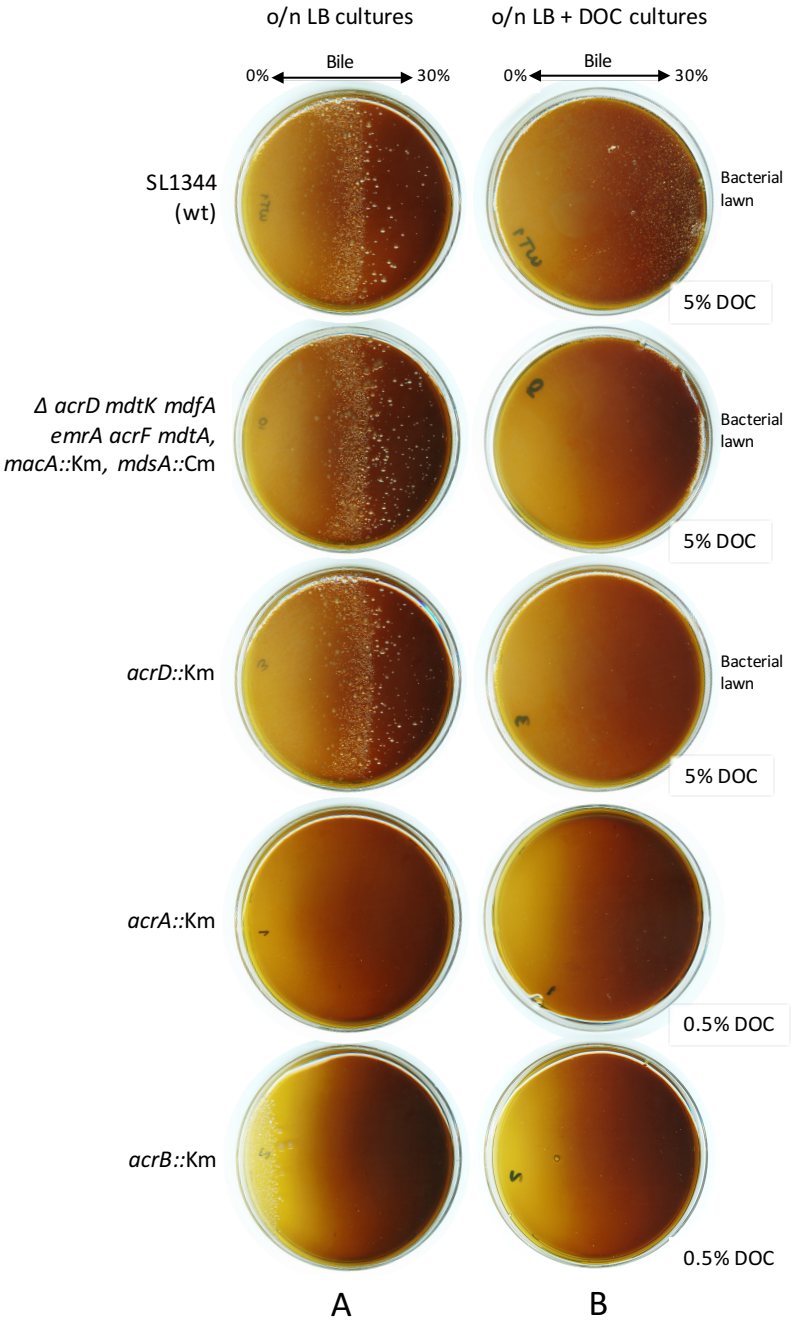

Figure C2.2. Adaptation assays in 0-30% ox bile gradient plates of mutants lacking efflux systems. Plates on the left side column (A) show the bile resistance levels of each strain cultured overnight in LB broth. On the right side column (B) the plates of the same strains cultured overnight in LB with sublethal concentrations of sodium deoxycholate

(DOC). In the wt, SV7636 (*Δ acrD mdtK mdfA emrA acrF mdtA, macA::*Km*, mdsA::*Cm), and the *acrD::*Km strain, a bacterial lawn appeared. The percentage of DOC used for adaptation is in the right side of the right column. The strain used as control was SL1344.

The results showed that it was evident that these efflux pumps did not have an additive effect in bile resistance, all of them showed a very similar level of resistance as that seen in the wild type strain (SL1344 *his*$^{+}$). Moreover, even the mutant that lacked all of the efflux pump systems but AcrAB (SV7636: *Δ acrD mdtK mdfA emrA acrF mdtA, macA::*Km*, mdsA::*Cm) showed the same bile resistance level than the wt strain (Figure C2.2 A).

Once the levels of resistance of these mutants were determined, exponential phase cultures of these strains were incubated overnight with sublethal concentrations of sodium deoxycholate (5% DOC for most strains and 0.5% for *acrA* or *acrB* mutants). The following day, dilutions of these cultures were plated in ox bile gradient plates and incubated overnight at 37°C. Results demonstrated the ability of these mutants, with multiple efflux systems impaired, to adapt to bile (Figure C2.2 B).

Finally, it is important to note that two efflux pumps require the subunit AcrA as part of its system: AcrAB and AcrAD [75,81]. Nevertheless, an *acrD* mutation does not affect in bile resistance nor adaptation (Figure C2.2). These results corroborate the importance and specificity of AcrAB-mediated efflux in bile resistance and adaptation.

Lack of AcrAB suppresses bile hyperresistance

Previous studies made in our laboratory had described mutants that display bile hyper-resistance phenotypes. The findings about the importance of the AcrAB efflux system in bile resistance and adaptation raised the question of whether this cellular function might be likewise involved in bile hyperesistance. The idea of the experiment was to test if lack of the AcrAB efflux system affected the levels of bile resistance in such mutants. For this purpose, an *acrA::*Km allele was transduced with P22 HT into three hyperesistant strains: (i) BR6, a mutant carrying a deletion in the *yrbK* gene, involved in

lipopolysaccharide transport [88]; (ii) an *asmA* mutant lacking the outer membrane protein AsmA [123]: (iii) a *prc* mutant lacking Prc periplasmic protease [124].

Exponential cultures of *acrA yrbK, acrA asmA*, and *acrA prc* were diluted 10 fold and aliquots of 100 µl were plated on 0-20% ox bile extract gradient plates. The plates were incubated overnight at 37°C and growth was monitored afterwards.

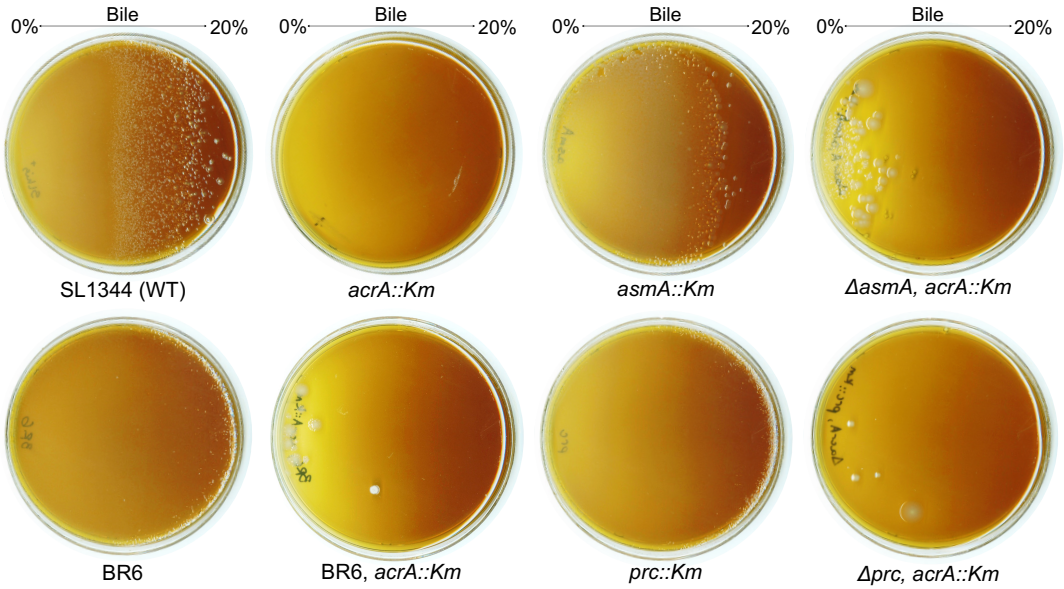

Figure C2.3. Suppression of bile hyperesistance by lack of AcrAB. Assays were carried out on 0-20% ox bile extract gradient plates.

The results displayed on figure C2.3 show that lack of AcrAB suppresses the bile hyperesistant phenotypes of *yrbK, asmA,* and *prc* mutants, as shown by disappearance of the bacterial lawn. These findings further outline the importance of the AcrAB efflux pump to maintain homeostasis in the bacterial cell: even though the alterations that render *yrbK, asmA,* and *prc* mutants bile-resistant seem to be unrelated to AcrAB, lack of the AcrAB efflux pump made them bile-sensitive.

Activation of acrAB expression by sodium deoxycholate

The relevance of AcrAB-mediated efflux in both bile resistance and bile adaptation led us to study the expression pattern of the *acrA* and *acrB* genes during the bile adaptation process. For this purpose, transcription analysis was performed in the presence of sub-

lethal concentrations of sodium deoxycholate (DOC). The *acrA* and *acrB* mRNA levels were determined by qRT-PCR in exponential and stationary cultures grown with 5% DOC. The strains under study were SV7636 (*ΔacrD ΔmdtK ΔmdfA ΔemrA ΔacrF ΔmdtA ΔmacA::*Km*, ΔmdsA::*Cm) and the wild type strain (SL1344). In the wild type, the expression levels of efflux pumps other than AcrAB were also determined (Table C2.1).

Table C2.1. Relative expression values of different efflux pump genes obtained from transcriptomic analysis (qRT-PCR) performed in the wt (SL1344) and SV7636 (*Δ acrD mdtK mdfA emrA acrF mdtA, macA::*Km*, mdsA::*Cm) strains grown in LB and LB 5% DOC and in both exponential and stationary phase. The values are expressed as the fold change in expression when compared with the wild type strain grown on LB. Data are normalized to the RNA levels obtained in the wild type strain cultured in LB (value for the wild type = 1). Data are from 3 independent experiments.

Wild type strain (SL1344)		
	Relative expression values (fold change) in LB 5% DOC	
Gene	Exponential phase	Stationary phase
acrA	14	4.5
acrB	10	2
acrD	2.5	2
acrF	1	4
emrA	2.5	0.9
macA	2	1
mdfA	2	7
mdsA	2	3
mdtA	2.5	2
mdtK	3	3
SV7636 strain (*Δ acrD mdtK mdfA emrA acrF mdtA, macA::*Km*, mdsA::*Cm)		
	Relative expression values (fold change) in LB 5% DOC	
Gene	Exponential phase	Stationary phase
acrA	12	5.5
acrB	12	3

Most efflux pumps appear to be up-regulated in exponential phase in the presence of DOC (Table C2.1). Notably, the *acrA* and *acrB* genes showed a gene expression increase of 10-fold or more. The results are similar in the 8 efflux pump mutant strain SV7636 (*Δ acrD mdtK mdfA emrA acrF mdtA, macA::*Km*, mdsA::*Cm), with a 12-fold increase in gene expression for both genes. Table C2.1 includes also additional efflux pump genes, whose expression was analyzed in the wild type: *acrF*, reported to export bile [75,81] showed no

increase in expression, while *macA*, not involved in bile transport [75,81], showed a 2-fold increase in expression in the presence of DOC. These results are presented in figure C2.4 as well, where a representation of the differences in gene expression is shown in a column chart. Moreover, the additional genes coding components from efflux pumps reported to export bile and sodium deoxycholate (DOC) [75,81] displayed slight increases in expression in the presence of DOC: ≈2.5-fold for *acrD, mdtA* and *emrA*. On the other hand, genes encoding efflux pumps not involved in bile transport also showed an increased expression with DOC: ≈2-fold for *mdsA* and *mdfA*, and ≈3-fold for *mdtK* (Table C2.1).

In stationary cultures, which have already adapted to sodium deoxycholate and/or bile, it is noticeable that the increase in the expression of the *acrA* and *acrB* genes is lower than in exponential phase, with values ranging from 2- to 6-fold. Moreover, *acrF* showed a 4-fold increase with DOC while *macA* did not change its expression (Table C2.1 and Figure C2.4). The other genes analyzed presented different expression patterns: *acrD* and *mdtA* ≈2-fold, *emrA* decreased its expression to ≈0.9-fold, *mdsA* and *mdtK* ≈3-fold, and *mdfA* increased up to ≈7-fold (Table C2.1).

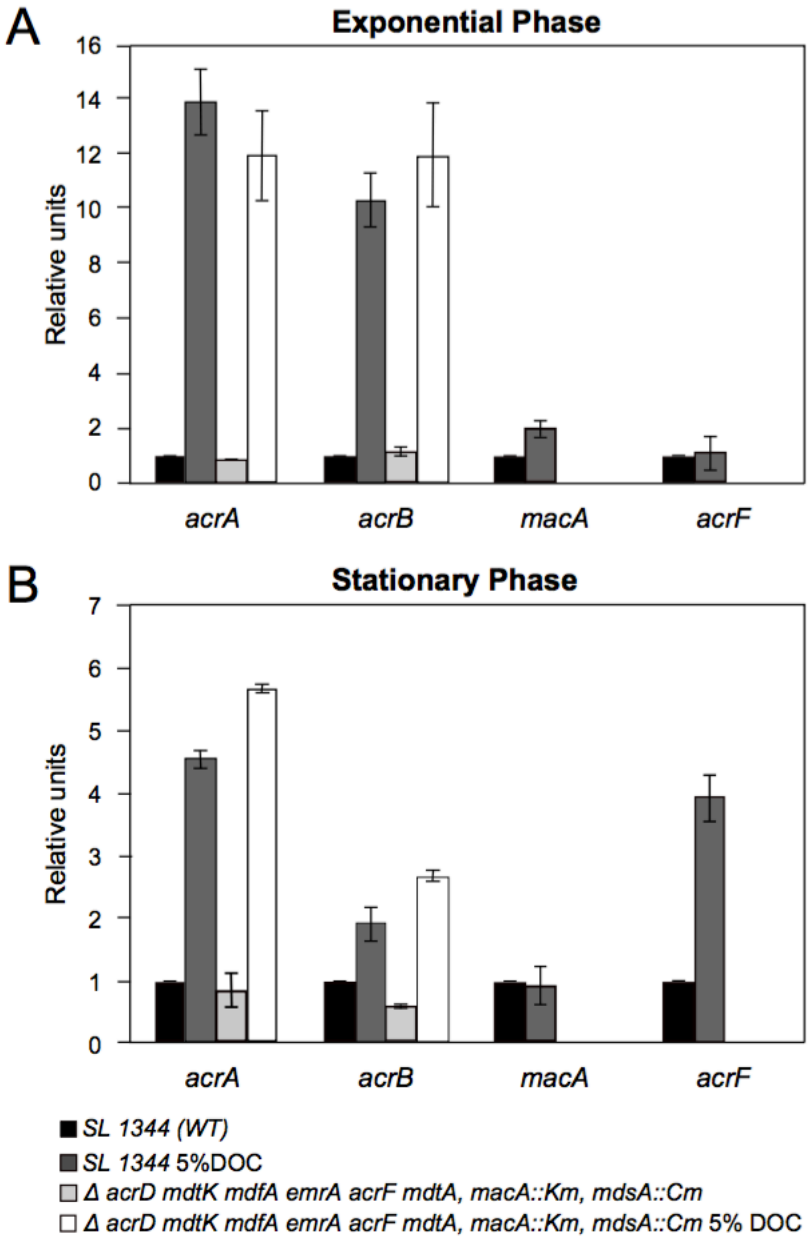

Figure C2.4. Transcriptional analysis of the *acrA*, *acrB*, *macA* and *acrF* genes in the WT SL1344 strain and the SV7636 strain (*Δ acrD mdtK mdfA emrA acrF mdtA, macA::*Km, *mdsA::*Cm) in cultures with and without DOC in exponential phase (A) and in stationary phase (B). Data are normalized to the RNA levels obtained in the wild type strain cultured in LB (value for the wild type = 1). Data are from 3 independent experiments.

The results obtained for *acrA* and *acrB* expression levels in exponential growth phase are similar to those obtained by other authors: a chromosomal fusion) of the *acrAB* promoter fusion to the *luc* gene (without disrupting the *acrAB* genes) showed an eightfold induction by bile [122]. Additionally, Usui *et. al.* (2011) found that bile-resistant strains of *S.enterica* serovar Pullorum showed high expression of *acrAB* in the presence

of sodium deoxycholate while strains susceptible to DOC had low expression, and enhancement of *acrAB* expression resulted in the acquisition of bile resistance in susceptible strains [125]. In our case the increase in *acrA* and *acrB* expression in exponential cultures led to bile adaptation. In addition, these observations suggest that *acrAB* expression is crucial at early stages of bile adaptation, but when the culture adapts this increase in gene expression may not be needed anymore.

Single cell analysis of AcrAB-mediated efflux

The temporal dynamics of *acrAB* expression raised the question on whether it coincided or not with the activity of the AcrAB efflux pump during the adaptation process. To address this question, AcrAB efflux activity was monitored in individual cells by flow cytometry using ethidium bromide (EtBr), a common substrate of bacterial efflux pumps [105,126,127]. The strains analized were the wild type (SL1344) and SV7636 (*Δ acrD mdtK mdfA emrA acrF mdtA, macA::*Km*, mdsA::*Cm), cultured in LB and LB + 5% DOC (bile adaptation conditions) (Figure C2.5).

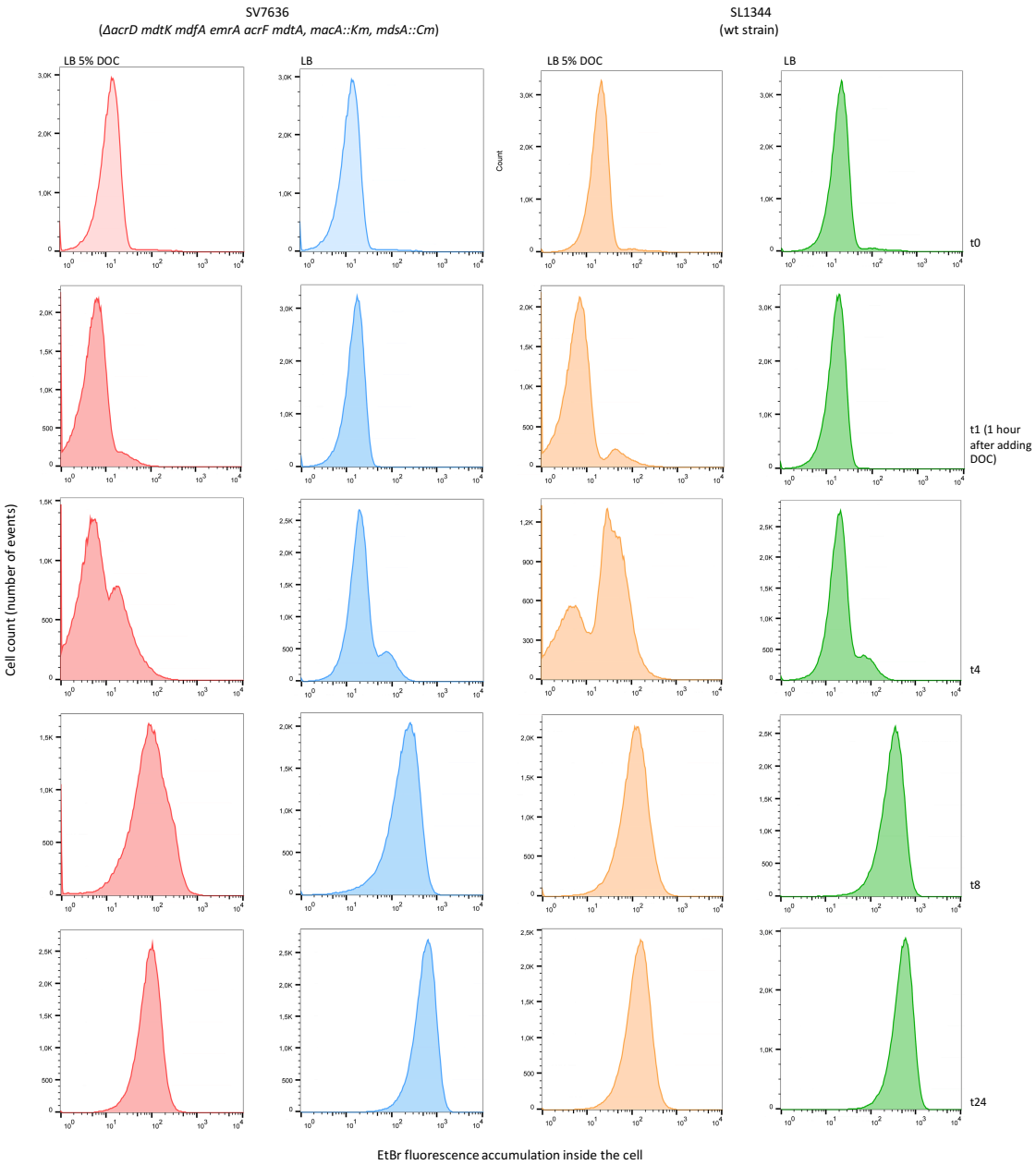

Figure C2.5. Single cell analysis of AcrAB-mediated efflux of sodium deoxycholate during a 24 h period. EtBr fluorescence of SV7636 (red and blue histograms) and SL1344 wt (orange and green histograms) cultures in LB and EtBR (1 µg/mL) with and without 5% DOC over time (0-24h after adding 5% DOC).

Figure C2.5 shows the dynamics of ethidium bromide fluorescence over time. The two columns on the left (red and blue histograms) correspond to the SV7636 strain, which lacks all efflux systems but AcrAB, grown in LB and LB 5% DOC. The two columns on the right show EtBr fluorescence in single cells of a wild type (SL1344) culture grown in LB and LB 5% DOC (orange and green histograms). Changes in EtBr fluorescence values are

portrayed from top to bottom. For each treatment and time point, 100,000 cells were scrutinized.

In exponential phase cultures (t0, t1, and t4 in Figure C2.5), a series of interesting observations were made. In t1 (one hour after sodium deoxycholate addition) a decrease in EtBr fluorescence was detected for both the wild type and the SV7636 strain compared with the LB cultures at the same time point, suggesting an increase in efflux. The fact that the behavior of SV7636 (which lacks all efflux systems except AcrAB) was similar to that of the wild type suggests that AcrAB plays a major role in bile and/or sodium deoxycholate elimination in the first stages of adaptation to bile. This interpretation is coherent with the pattern of *acrAB* gene expression (Figure C2.4). Four hours after the addition of sodium deoxycholate to the cultures, the ethidium bromide fluorescence within the cells starts to increase, more rapidly in the wild type strain than in SV7636 (*ΔacrD ΔmdtK ΔmdfA ΔemrA ΔacrF ΔmdtA macA::*Km *mdsA::*Cm). This observation suggests that the adaptation process is faster in the wild type. When grown in LB, both strains showed similar or identical behavior: low accumulation of EtBr during exponential growth. Altogether, these observations suggest that during exponential growth the efflux pumps are active independently of the presence of sodium deoxycholate but the efflux activity is higher in the presence of either bile or DOC.

In stationary phase, the flow cytometry histograms showed a different pattern of ethidium bromide fluorescence. In t8 (8 hours after DOC addition), both strains showed a similar pattern of EtBr accumulation, which increased with time. These cultures, which have been growing in 5% DOC, are already adapted at this point. After twentyfour hours of growth with sodium deoxycholate, the EtBR fluorescence histograms are similar to those obtained at t8, perhaps indicating a decrease in efflux activity. When compared with the strains cultured in LB, it can be perceived that in LB EtBr fluorescence increases even more inside the cells, which suggests that a stronger decrease in efflux activity occurs at this stage when no bile nor DOC are present. This difference in EtBr fluorescence inside the cells after 24h of growth with and without DOC is even more clear in figure C2.6, where the histograms of all cultures grown in both conditions are plotted together: the bile-adapted cultures of SL1344 and SV7636 show less EtBr

accumulation when compared with the cultures without sodium deoxycholate. Furthermore, it is noticeable that, upon DOC treatment, the wild type strain accumulates less EtBr than the SV7636 strain (*ΔacrD ΔmdtK ΔmdfA ΔemrA ΔacrF ΔmdtA, macA::*Km*, mdsA::*Cm). On the other hand, both strains show similar histogram patterns for EtBR accumulation when cultured in LB alone. Comparison of both treatments demonstrates that the presence of DOC keeps the efflux active, independently of the growth phase.

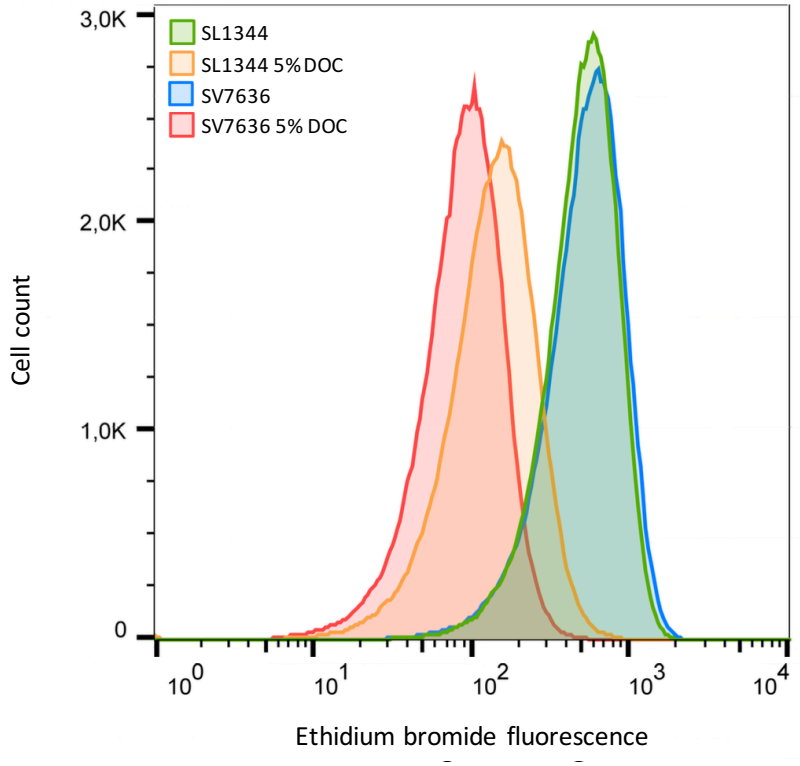

Figure C2.6. Single cell analysis of sodium deoxycholate AcrAB-mediated efflux after 24 hours. EtBr fluorescence of SV7636 and SL1344 wt strains cultures in LB and EtBR (1µg/mL) with and without 5% DOC after 24 h.

To better observe the differences between each strain and each treatment regarding ethidium bromide accumulation over time, EtBr mean levels within the cells were determined for all time points studied and represented in a scatter plot (Figure C2.7).

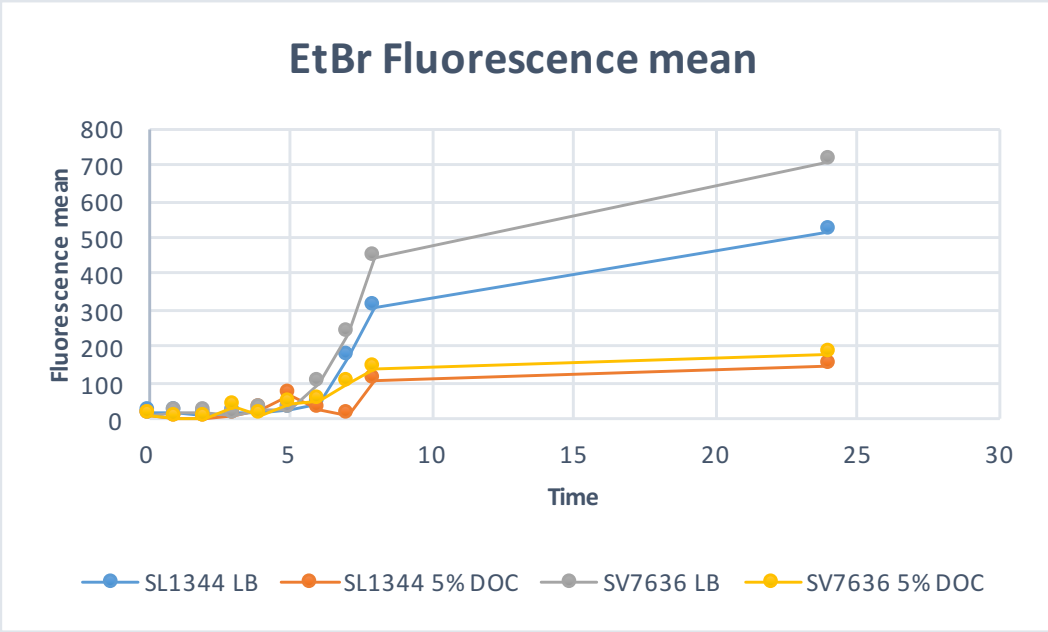

Figure C2.7. EtBr fluorescence means over time. Values were obtained from cytometry assays with the eight efflux pump mutant SV7636 (*ΔacrD ΔmdtK ΔmdfA ΔemrA ΔacrF ΔmdtA, macA::Km, mdsA::Cm*) and the wild type strain SL1344. A representative experiment is shown.

Figure C2.7 confirms that during exponential growth efflux is active in a sodium deoxycholate-independent manner, as indicated by the low ethidium bromide levels detected in all cultures. After 6-7 hours, as the samples began to enter stationary phase, cultures of both strains in LB started to accumulate EtBr at a higher rate than the cultures in LB 5% DOC. By hour eigth of incubation, cultures of SL1344 and SV7636 in LB showed EtBr accumulation around 300 and 450 fluorescence units, respectively. On the contrary, when both strains grew in the presence of 5% DOC, moderate accumulation of EtBr was detected (150 units for the SV7636 strain and around 100 for the wild type). The differences between LB and LB + DOC increased further after 24 h, suggesting that cultures in LB decrease their efflux activity upon entrance into stationary phase. In contrast, cultures grown with 5% DOC seem to keep their efflux active irrespectively of the growth phase.

CHAPTER 3: A STUDY OF MECHANISMS OF BILE RESISTANCE *IN VIVO*

Characterization of S. enterica populations present in the gallbladder of BALB/c mice (typhoid fever animal model).

BALB/c mice are particularly susceptible to the *S. enterica* serovar Typhimurium infection because they are homozygous for a mutation in the *Bcg* locus, also known as *Ity* or *Lsh*. This gene codes for the Nramp1 (natural-resistance-associated macrophage protein 1) protein, which controls the innate resistance or susceptibility of mice to infection with a group of unrelated intracellular microorganisms that include *Salmonella, Leishmania* and *Mycobacterium* [128,129]. To analyze the *S. enterica* populations that colonize the gallbladder upon systemic infection, 19 BALB/c mice were orally infected with 10^8 colony forming units (c.f.u.) from overnight anaerobic cultures of the wild type strain (SL1344). Five days later, the mice showed the previously described clinical disease symptoms. At this stage the mice were euthanized and the gallbladders were recovered.

The gallbladders were homogenized, and serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were prepared with NaCl 0.9%. To select Salmonella isolates and avoid growth of other intestinal bacteria, the dilutions were plated on LB agar + streptomycin 200 μg/l, and incubated overnight at 37°C (SL1344 is resistant to low concentrations of streptomycin). The following day, the colonies were counted. Table C3.1 shows the number of colony-forming-units (c.f.u.) present in the gallbladder of each mouse (applying dilution factors to the calculation when necessary).

Table C3.1. Estimated number of c.f.u. recovered from the gallbladder of BALB/c mice.

Mouse	Number of c.f.u. recovered from the gallbladder
1	63
2	548
3	105
4	475
5	6694
6	51
7	31204
8	37
9	1042
10	222
11	2858
12	203400
13	182
14	422
15	202500
16	1599000
17	1018
18	2783
19	11203

Although BALB/c mice are genetically identical and the experiment consisted in inoculating all the mice with the same strain and with identical numbers of cells, the numbers of c.f.u. present in the gallbladder extracts show high variability from one mouse to another. This variation could have several causes. For instance, because the inoculation was done orally, the number of bacteria that ultimately reached the digestive system might be different in each mouse. Non-genetic variability in each mouse might alter the ability to respond to the infection. Another source of variation could be stochastic variation in the number of *S. enterica* cells able to invade and replicate within the different gallbladder microenvironments.

Certain plates used for the isolation of gallbladder Salmonellae contained isolated colonies while others contained a bacterial lawn. Plates containing isolated colonies were replicated with sterile velvets to LB agar containing high concentrations of ox bile (from 12% to 20%). This experiment permitted to distinguish bile-resistant mutants from

isolates sensitive to bile, which were by far the most abundant type (Figure C3.1). The four lawns obtained were also replicated, and provided 1, 32, 49, and 122 mutants; a ratio between bile-adapted and bile-resistant isolates could not be calculated in those gallbladders.

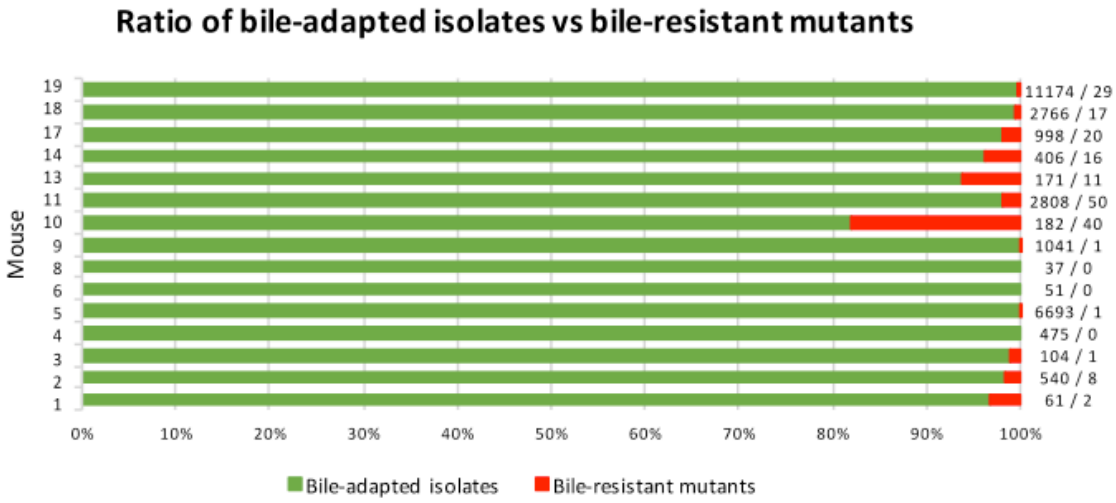

Figure C3.1. Characterization of *S. enterica* BALB/c mice gallbladder populations: ratio between bile adapted and bile resistant mutant isolates recovered from each BALB/c mouse gallbladder. The ratio is represented in percentage values. On the right side of the chart, the actual numbers of bile-adapted isolates vs bile-resistant mutants are shown.

The main conclusion from these experiments was that colonization of the gallbladder by Salmonella is made possible mainly by adaptation (in other words, without mutation). However, bile-resistant mutants also appear at low frequencies. These observations are in agreement with previous studies of bile resistance in vitro which also suggested that non-mutational adaptation to bile was more frequent than mutation [88]. An additional observation is that the numbers of bile-resistant mutants show significant differences from one gallbladder to another, and that correlations do not seem to exist between the number of mutants and the total number of c.f.u. This observation suggests that Salmonella may grow in the gallbladder for a significant number of generations in the gallbladder, and that Luria & Delbrück fluctuation may occur. Unfortunately, this

hypothesis cannot be subjected to statistical analysis due to the lack of an outgroup that might serve as control.

Characterization of bile-resistant mutant derivatives isolated from the gallbladder of BALB/c mice.

Ten bile-resistant mutants of independent origin (each isolated from a different mouse gallbladder) where chosen for full genome sequencing. The mutants were propagated from colonies grown in the original plates inoculated with gallbladder homogenates. Therefore, the mutants had not arisen *after* recovery from the gallbladder. Genome sequencing employed Roche 454 FLX+ technology on a GS FLX titanium system. Sample preparation included gDNA isolation by phenolic extraction and ethanol precipitation. Emulsion PCR and 454 pyrosequencing were performed following the manufacturer's instructions and 1,031,375 reads with an average read length of 810 basepairs were obtained, totaling 1,000Mb.

Genome sequencing was followed by alignment with the *S. enterica* SL1344 genome sequence (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/STmSL1344.dbs) to identify DNA sequence differences. The alignment tool used was BWA (Burrows-Wheeler Alignment), which consists of three algorithms. In our case, the BWA-MEM algorithm was employed (Li and Durbin, unpublished). The genome of the laboratory stock of *S. enterica* SL1344 was also sequenced as a control. Table C3.2 describes the mutations found in the mutants isolated from murine gallbladders.

Table C3.2. Mutations present in the genome of bile resistant derivatives of *S. enterica* SL1344 isolated from BALB/c mice gallbladder.

Mutant	Genome position	Locus affected	Mutation	Location of the mutation	Predicted mutation change	Cellular function affected
1	708234	*rlpB*	G→T	Nucleotide 287	Ala→Glu	Lipopolysaccharide transport
2	2326694	*yejM*	A→T	Nucleotide 58	His→Leu	Unknown
3	4589340	*dipZ*	C→A	Nucleotide 328	Ala→Ser	Copper tolerance and cytochrome c biogenesis
4	153536	*ftsQ*	A→T	Nucleotide 1	Met→Leu (start codon change)	Cell division
5	4072585	Intergenic region STnc400 (sRNA) and STM3845 (hypothetical protein)	Deletion of 1 nt (A)	Base pair 4072585	Unknown	Unknown
6	3660275	*dam*	T→A	Nucleotide 771	Synonym mutation	DNA methylation
7	994451	*ftsK*	Deletion of 36 nt	Base pairs 544-579	Loss of 12 amino acids	Cell division
7	1306516	STM1268	C→T	Nucleotide 107	Ala→Val	Unknown
7	3118294	*ygcF*	A→C	Nucleotide 398	Val→Gly	Unknown
7	3216955	Intergenic region STM3034 (hypothetical protein associated with virulence) and STM3036 (hypothetical protein)	G→A	Nucleotide 3216955	Unknown	Unknown
8	3505277	*yhbG*	G→A	Nucleotide 175	Ala→Thr	Unknown
9	2728613	Intergenic region *gogB* (Type III secretion system effector protein) and STM2585 (hypothetical transposase)	Insertion of 6 nt	After nucleotide 2728613	Unknown	Unknown
10	4162532	Intergenic region *yifK* (probable amino acid permease) and GlmZ (sRNA)	Deletion of 15 nt	Base pairs 4162533-4162547	Unknown	Unknown

The following observations may be relevant:

a. Mutant 1 shows a nucleotide substitution in *rlpB*, a gene that encodes a lipoprotein B precursor. A similar point mutation (transversion) has been previously reported in a study that isolated spontaneous bile resistant mutants from agar plates containing high (lethal) ox bile concentrations [88]. Even though this protein has not been widely studied in *S. enterica*, its *E. coli* homolog has been described to form a protein complex with the product of the *imp* gene (Imp/RlpB) that is responsible for LPS transport to the outer membrane. In addition, in *E. coli, rlpB* appears to be essential for cell viability [130]. Interestingly, this gene has been reported to be induced by bile [131].

b. Mutant 2 shows a point mutation (transition) in *yejM,* a gene that remains poorly known in *S. enterica* and may encode a hypothetical sulphatase. Transcription of *yejM* is induced by bile [131]. Its homolog in *E. coli* is classified as an inner membrane protein and its deletion is lethal. YejM might be involved in cell permeability and lipid A production [132,133].

c. Mutant 3 harbors a transversion in the *dipZ* gene, which encodes the DsdB thiol-disulfide interchange protein. In *E. coli*, studies have shown that DsdB is a cytoplasmic membrane protein (more specifically, a periplasmic protein disulfide bond isomerase) that transfers reducing power from the cytoplasm to the periplasm to aid in the formation of disulphide bonds and c-type citochromes [134,135]. In addition, [136] have suggested that this gene might have a role in copper tolerance.

d. Mutants 4 and 7 harbor mutations that involve cell division factors (*ftsQ* and *ftsK*) and show increased expression in the presence of bile [131]. In the case of mutant 4 there is a transversion that changes the *ftsQ* gene start codon from ATG (methionine) to TTG (leucine), which is the second most used start codon in *E. coli* [137]. Further analysis will be required to ascertain whether translation of the mutant mRNA actually uses the new triplet as start codon. Mutant 7 presents a deletion of 36 nt that results in the loss of 12 amino acids. Several studies made in *E. coli* regarding these division factors have demonstrated that FtsQ and FtsK both belong to a group of 9 essential proteins that participate during Gram-

negative bacteria in septum formation. The remaining proteins are FtsA, FtsI, FtsL, FtsN, FtsW, FtsZ, and ZipA and all are localized in the cell septum [138,139]. A previous study from our laboratory identified another cell division factor whose synthesis is induced by bile. This gene is is currently annotated as yiiU in S. enterica, and is an homolog of E. coli zapB [88].

e. Four independent mutants (5, 7, 9 and 10) show mutations in intergenic regions. Interestingly, in candidates 5 and 10, the genomic regions where the mutations are present have been reported to be highly induced by bile [131], particularly, the small RNAs STnc400 and GlmZ, respectively.

f. Mutant 6 shows a synonymous nucleotide substitution in the DNA adenine methylase gene *dam.* This result is curious as *dam* mutations causes pleiotropic defects which include increased bile sensitivity [63,85,140,141].

g. Mutant 8 harbors a mutation in a gene of known function which is induced by bile [131], *yhbG*. This gene has not been described in *S. enterica* but its *E. coli* homolog appears to be essential, and might have a role in cell envelope integrity [142]. This gene is predicted to be an ABC transporter [143,144].

DISCUSSION

The ability of the foodborne pathogen *Salmonella enterica* to survive in disparate environments (soil, water systems, slaughter houses, food processing plants, and animal hosts, among others) outlines the importance of the survival strategies of the bacterium. Such a knowledge can be useful to identify potential therapeutic targets to combat *S. enterica* infections and/or outbreaks.

This study has investigated the mechanisms that *S. enterica* employs to survive bile, a gastric secretion that has antimicrobial activity due to the presence of bile salts. When *S. enterica* encounters bile, one of two events can happen depending on the bile concentrations: (1) If the concentration of bile is high, the bacteria will die upon homeostasis disruption involving cell membrane damage, denaturation of proteins, and DNA damage; (2) If the concentration of bile concentrations is low, the bacteria will survive and will trigger a combination of stress responses that involve major changes in the expression pattern. This complex response will allow the bacteria to survive in the presence of even higher concentrations of bile. The latter phenomenon is known as adaptation [86,88,87].

Under laboratory conditions, *S. enterica* adaptation to bile can be monitores as an increase in the bile resistance level after previous exposure to sublethal concentrations of bile or individual bile salts (e. g, sodium deoxycholate, DOC). Adaptation is reversible, and bile resistance decreases back to the original level if bile or DOC is removed from the medium (figure D1).

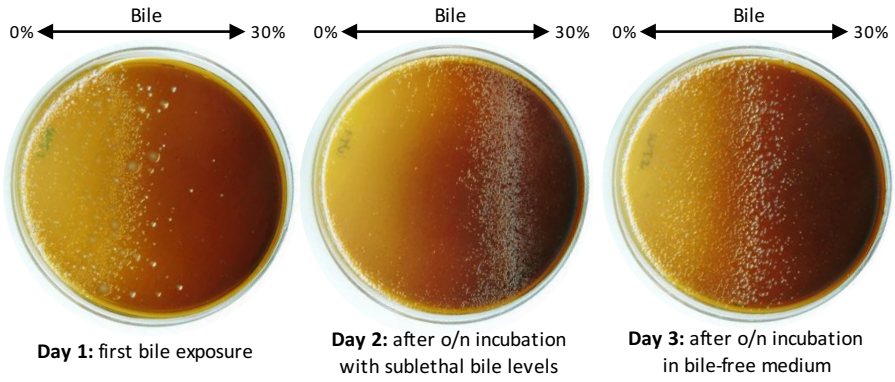

Figure D1. Assay of adaptation to bile on bile gradient plates (0-30%) in *S. enterica* serovar Typhimurium strain SL1344. From left to right, the first plate shows the bile resistance level of a culture not exposed to bile. The central plate shows the increase in

the bile resistance level after after overnight incubation with a sublethal concentration of bile. Finally, the plate on the right shows how bile resistance returns to the initial level after removal of bile from the medium (overnight incubation of the culture in LB without bile).

This reductionist visualization of bile adaptation may serve as a model for events occurring during infection of animals. When bile is released into the intestine to aid in digestion, the concentration may be low enough to permit survival, and high enough to turn on the responses that permit adaptation. If the infection proceeds to the systemic stage, Salmonella cells that have adapted to bile may have higher chances of survival in the liver and especially in the gallbladder, where the concentration of bile salts is extremely high [88].

Bile resistance can be also acquired by mutation, and the fact that bile salts are DNA-damaging agents have raised the possibility that adaptation to bile might involve bile-induced mutagenesis [63,64]. The fact that the gallbladder contains high concentrations of bile salts, combined with the ability of *Salmonella* to remain in the gallbladder for long periods, may support this possibility. Even though bile salts are not strong mutagens, the frequency of mutation is often proportional to the dose of the mutagen, which is the product of concentration by time of exposure. Based on these considerations, one of the objectives of this Thesis was to ascertain whether colonization of the gallbladder involved mutational or non-mutational adaptation.

Although a number of genetic loci that contribute to *S. enterica* bile resistance are known, their specific role in bile adaptation had not been investigated previously. In order to ditinguish loci involved in bile resistance from loci involved in non-mutational adaptation, a collection of bile-sensitive mutants of *S. enterica* was subjected to adaptation trials. For this purpose, we employed the adaptation assay depicted in Fig. D1 using concentrations of bile appropriate for each strain.

After determining minimal inhibitory concentrations (MICs) of sodium deoxycholate (DOC), each strain was grown overnight with a concentration of DOC sublethal for the strain. The following day, the cultures were plated on 0-30% bile gradient agar and

incubated overnight at 37°C. We were surprised to find out that most of the bile-sensitive mutants under study showed enhanced bile resistance, meaning that these mutants were still able to adapt (Table C1.1 and Figure C1.1). It s remarkable that functions involved in DNA methylation (*dam*), virulence (*phoP* and *wecD*), cell division (*damX* and *zapB*), response to environmental stresses like starvation, oxidation and low pH (*rpoS*) [73,107,108,110,113,114,116] appear to be dispensable for bile adaptation. On the contrary, efflux functions appear to be critical, as indicated by the observation that *acrA, acrB,* and *tolC* mutants are not able to adapt.

The AcrB protein belongs to the resistance nodulation division (RND) type of efflux systems, which are found throughout the *Archaea*, *Bacteria*, *and Eukaryota* domains. In Gram-negative bacteria, RND efflux systems have a component embedded in the inner membrane, which forms a complex with two other proteins, a periplasmic adaptor protein (in this case AcrA) and an outer membrane channel (TolC) (Figure I7) [145,146]. In general, RND efflux systems transport a wide range of substances outside the cell (antibiotics, dyes, detergents, host derived molecules, etc.) [147,148]. in *S. enterica*, these compounds include bile salts, and it is known that expression of *acrAB* and *tolC* genes is induced by bile [79,84,87,122]. It is also known that the AcrAB efflux system is absolutely required for bile resistance [38,122,149,150]. Regarding the outer membrane pore, TolC, Prouty et. al (2002) showed that, like *acrAB* mutations, *tolC* mutants also bile-sensitive [151]. This is not surprising as mutations in *tol* genes destabilize the membrane, allowing greater access of bile salts into the bacterial cell [38].

Despite intensive research on the AcrAB-TolC complex, its role in adaptation to bile has not been investigated previously. An exception was a study by Lacroix et al. showing that a bile-sensitive mutant, later identified as an *acrB* mutant, acquired increased detergent resistance after previous incubation on cholic acid [149]. This observation, which may suggest adaptation, is questioned by our finding that lack of the AcrAB-TolC efflux pump renders Salmonella unable to adapt to bile. One potential (but unlikely) explanation for the discrepancy is that that Lacroix et. al worked with cholic acid, while in our adaptation experiments we used ox bile and the sodium deoxycholate. The reason why this explanation seems unlikely is that all bile salts seem to have similar

antibacterial activities (Prieto et al. 2006).

A search for multicopy suppressors of the bile-sensitive phenotype in the absence of AcrAB-TolC strenghtened our view that efflux is essential for adaptation: most of the pBR328 derivatives that restored bile resistance and permitted bile adaptation harbored efflux pump components such as AcrF, AcrE and AcrB (Table C1.3).

The *acrE* and *acrF* genes encode the RND efflux system AcrEF, and AcrF is homologous to AcrB in *S. enterica*, with around 81% of amino acid identity and similar substrate specificity [81]. It has been reported that AcrEF might be required for drug resistance when AcrAB is impaired in *S. enterica* [152]. Evidence of this kind was found by Olliver et al. (2005) in the case of antibiotic resistance: an otherwise sensitive *acrB* mutant was found to have elevated fluoroquinolone tolerance due to increased expression of *acrEF*, in turn caused by an insertion sequence integrated upstream of the *acrEF* operon [153]. A similar case was observed by Horiyama et. al (2010): the enhanced expression of *acrEF* in an *acrB* background increased resistance levels to a large number of antibacterial compounds including sodium deoxycholate [75]. An analogous situation might occur in our case: increased expression of *acrEF* could rescue the bile-sensitive phenotype of the *acrA* mutant.

Because both the adaptation survey performed with bile-sensitive mutants and the multicopy suppressor screen showed the relevance of active efflux for adaptation to bile, (Table C1.3), subsequent experiments were focused on the role played by efflux pumps in adaptation to bile. The involvement of the remaining efflux systems reported to exist in *S. enterica* [81] was thus investigated. Figure D2 shows the distribution of the corresponding loci in the genome *S. enterica* serovar Typhimurium, along with their transcriptional orientation and identity percentage with their *E. coli* homologs.

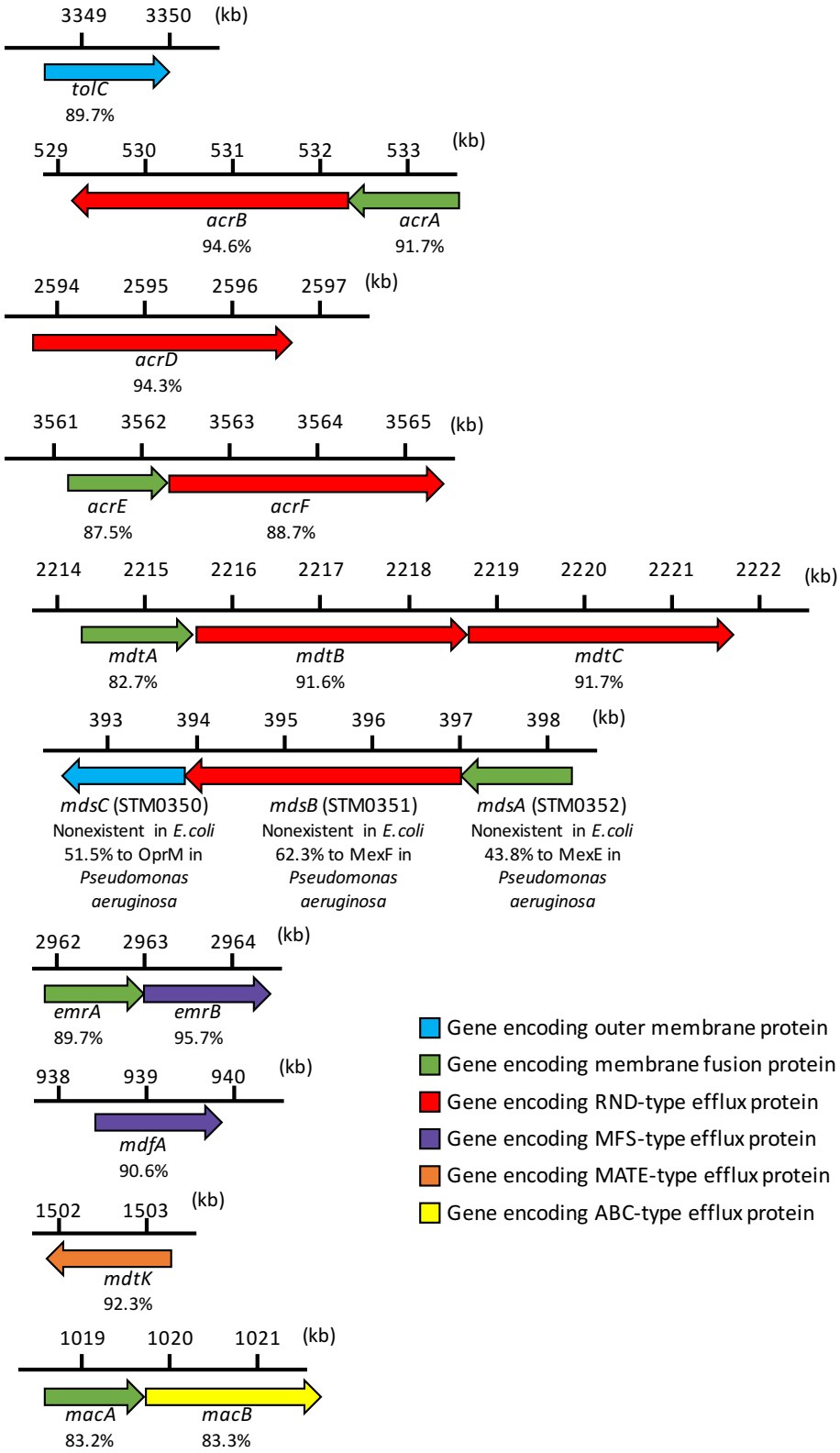

Figure D2. Drug efflux genes encoded on the *S. enterica* serovar Typhimurium genome. Positions of genes encoding putative drug transporters, outer membrane proteins and membrane fusion proteins are indicated in the chromosome of *S. enterica* serovar Typhimurium strain LT2 [6]. Arrows indicate the direction of transcription. Amino acid identity between homologous proteins in *S. enterica* and *E. coli* are indicated as numbers under the genes. Reproduced from [81].

Using the available information concerning the efflux systems present in *S. enterica,* mutants lacking at least one of the genes of each efflux system were constructed. Minimal inhibitory concentrations (MICs) of sodium deoxycholate (DOC) were then determined. Remarkably, all mutants but *acrAB* showed DOC resistance levels similar to that of the wild type (Figure C2.1). This was a surprise as several of these efflux pumps (including AcrAB) had been reported to export bile and bile salts (AcrAD, AcrEF, mdtABC, EmrAB) [75,81,154]. These results could be explained in two different ways:

1. Deletion of these genes might be unnoticeable by the presence of a functional AcrAB efflux system. It is known that AcrAB has an extremely wide specificity, exporting multiple types of antibacterial agents (with the exception of aminoglycosides), detergents, dyes, free fatty acids, and simple solvents [154–156]. Particularly, reconstitution assays of the purified AcrAB efflux system into proteoliposomes concluded that, after testing different ligands, the properties of AcrB were optimized for the exclusion of bile salts [78].
2. The deleted genes might not be expressed under the conditions of the experiment.

To probe the first possibility, a set of strains lacking multiple efflux pumps was constructed. These strains were tested for bile resistance and adaptation ability (Figure C2.2). The strains had from 2 to 8 efflux systems impaired, but AcrAB remained intact. The results on bile resistance levels were conclusive: even the strains that lack every efflux system but AcrAB had a bile resistance level similar to that of the wild type. Additionally, all these mutants were able to adapt to bile after incubation with sublethal levels of DOC. These results underline again the relevance of AcrAB-mediated bile efflux in the adaptation process.

The need of AcrAB in the transient increase of bile resistance levels (adaptation) raised the possibility that mutation of this efflux system might impair bile resistance and bile adaptation in bile-resistant mutants. The mutants chosen to address this question were (*yrbK*, *asmA::*Km and *prc::*Km), all carrying mutations that affected the structure of the cellular envelope. The results of these experiments showed that an *acrA* mutation

generated a bile-sensitive phenotype in the three backgrounds under study. This can be explained by admitting that some efflux systems, including AcrAB, may act synergistically with the permeability barrier [148]. In other words, the mechanisms of Gram-negative bacteria to survive when exposed to toxic compounds are mainly two (permeability barriers and active pumping out), and they must function together [147,157]. This means that the permeability barriers are insufficient to prevent influx of obnoxious compounds, and their action simply slows down uptake. The efflux pumps, which form multisubunit complexes by association with outer membrane channels (like TolC) and accessory proteins (AcrA) are thus necessary extrude toxic compounds directly into the medium, bypassing the outer membrane barrier [148]. Even if this complex mechanism has been so extensively described [78,147,157–160], it still comes as a surprise that mutants that show an exceptionally high resistance to bile rely so greatly in the bile extrusion role of AcrAB. This result only confirms the weight the efflux system has in conferring the bacteria the ability to survive in the presence of bile and to adapt to grow in the presence of increasing concentrations.

To monitor the expression patterns of each *S. enterica* efflux system during the bile adaptation process, qRT-PCR experiments were carried out under different conditions: with and without sublethal concentrations of sodium deoxycholate, and in exponential and stationary growth phase. The strains assayed were the wild type and the mutant lacking eight efflux systems (SV7636), and the results are shown in Table C2.1 and Figure C2.4. Relevant results were as follows:

1. In exponential phase, transcription of both *acrA* and *acrB* genes is highly activated by sodium deoxycholate; on the contrary, the expression level of other genes, including those which encode components of efflux systems reported to export bile salts, are unaffected or poorly induced by the presence of DOC

2. In stationary phase, the level of expression of *acrAB* decreases drastically while most of the other genes do not show remarkable changes, except *acrF* and *mdfA*.

These observations give further support to the view that AcrAB is fundamental for adaptation. It seems that the key event for adaptation occurs during the first encounter of the bacteria with bile, which leads to increased expression of genes that encode the efflux pump. As time passes by and adaptation takes place, activation of gene expression decreases. In stationary phase, it is curious to observe that the *mdfA* gene is so highly induced, even if the efflux system is not reported to have bile salts as a substrate [75,81]. On the other hand, the increased expression of *acrF* could make sense as AcrEF is somewhat similar to AcrAB, both in amino acid identity and in substrate specificity [81]. Activation of *acrEF* expression might indicate that in this growth phase, once the culture is adapted, this efflux pump might contribute to bile salt export along with AcrAB. The mutant lacking all efflux systems but AcrAB shows a similar pattern of gene expression to that seen in the wild type for both *acrA* and *acrB* genes. Since the mutant is able to adapt and grow, this result and the observations made in stationary cultures of the wild type strain suggest that the role of the two differentially expressed efflux systems (*acrEF* and *mdfA*) in stationary phase do not play a relevant role in bile salt export.

While the leading function of the efflux system AcrAB in the adaptation process is out of question, it was curious to observe how, in spite of such an importance, the expression of both genes greatly decreased in stationary phase, when the cultures are already adapted (Table C2.1, Figure C2.4). To obtain more information about how the AcrAB efflux system contributes to bile adaptation, AcrAB activity was monitored by flow cytometry using ethidium bromide (an AcrAB substrate) as a reporter. For this purpose, ethidium bromide accumulation was tested in the wild type and in the mutant lacking all efflux systems but AcrAB (SV7636) with and without 5% DOC. Ethidium bromide accumulation within individual cells changed with time, especially when in the presence of DOC. The results shown in Figure C2.5 provide several conclusions:

1. In the absence of sodium deoxycholate both strains showed a similar pattern of eithidium bromide accumulation.

2. Addition of DOC caused a decrease of EtBr accumulation within the cells of both strains, suggesting DOC-dependent activation of the efflux systems.

3. Even if DOC remained present in the medium, gradual accumulation of EtBr occurred. This trend was faster for the wild type than for the SV7636 strain. During this shift, two subpopulations appeared: one that accumulated EtBr and one that did not. Since adapted cultures seem to accumulate more EtBr than not adapted cultures, we hypothesize that these two subpopulations might correspond to bile-adapted bacteria (right peak) and bacteria that are still undergoing adaptation (left peak).

4. The bile adaptation process takes less than 8 hours after the addition of the bile salt sodium deoxycholate and ends with an increased intracellular EtBr level, but such levels never reach those from cultures never exposed to DOC. These observations may have two alternative explanations, which are not mutually exclusive. One is that, once cultures are adapted, the intracellular concentration of bile salts increases as the bacterium has already activated mechanisms that permit to endure the damaging effects of bile within the cells: activation of the RpoS-dependent general stress response, activation of the SOS system, DNA repair mechanisms, etc. This hypothesis is supported by the fact that these responses are elicited indeed by the presence of bile (Cano et al., 2002; Hernandez et al., 2012; Merritt & Donaldson, 2009; Prieto et al., 2006). An alternative possibility is that bile-adapted cultures may have remodeled their cellular envelope to significantly decrease influx of bile salts, thus permitting a decrease in AcrAB activity. Is has been previously shown that changes in the cellular envelope (LPS, charge, hydrophobicity, etc.) alter bile resistance levels in several bacterial species [57–60].

Figure C2.6 shows an overlap between the EtBr fluorescence histograms of the wild type and the mutant lacking eight efflux systems (SV7636), both in the presence and in the absence of DOC*. In cultures grown without DOC, accumulation of EtBr is higher, suggesting a decrease in AcrAB activity when DOC is not present. The cultures with DOC show less EtBr accumulation after 24 h, indicating that the efflux system remains active in a DOC-dependent manner. Figure C2.7 shows a comparison over time of the EtBr fluorescence mean in each strain and treatment. In this scatter chart, activation of the AcrAB efflux system can be indirectly monitored by EtBr accumulation. For both*

treatments, the efflux pump remained active in exponential phase, indicating that AcrAB activity at this stage is independent of the presence of DOC. On the other hand, as the culture grows and adaptation occurs, the chart shows how the efflux system remains more active in the presence of DOC. Also, in LB the mutant lacking most efflux systems (SV7636) accumulates more EtBr than the wild type, suggesting that the wild type strain exports EtBr more efficiently than the SV7636 strain. This may be easily understood if other efflux systems aside from AcrAB export DOC [75,154].

Taking into consideration the experimental data discussed above as well as information from the literature, we propose that AcrAB is the only cell function essential for adaptation, and that activation of *acrAB* expression may be crucial at early stages of adaptation. As soon as a critical amount of AcrAB is present in the cell, however, sustained AcrAB activity may be sufficient to confer bile resistance.

Because animal models are essential to understand pathogenesis [161] a goal of this Thesis was to test adaptation to bile in an animal model. BALB/c mice may be an imperfect model of typhoid fever but the relevance of this infection can make *in vivo* studies worthwhile, no matter if imperfect or reductionist. On the other hand, bile resistance may be a crucial trait in typhoid fever as the chronic carrier state may be a key feature for continued maintenance of the pathogen within human populations [162]. Chronic carriers intermittently shed the bacteria for a prolonged, ill-defined period of time in the local environment and thus may spread the disease in the community and maintain a reservoir of infection [163].

Our study of bile adaptation *in vivo* involved the analysis of populations of *S. enterica* isolated from BALB/c mice gallbladders 5 days after oral infection. The analysis consisted on counting the number of colony forming units recovered from each individual gallbladder as well as determining their bile resistance levels and the stability of their resistance. The number of c.f.u. present in each gallbladder was extremely variable (Table C3.1). More remarkably, bile resistant was transient in over 80% of the isolates. Because these isolates had been able to survive in the gallbladder, simple statistical analysis seems to support the conclusion that bile adaptation is a common mechanism

employed by *S. enterica* to endure the bactericidal action of bile upon gallbladder colonization.

Bile-resistant mutants were also isolated from the gallbladder of BALB/c mice, and full genome sequencing identified the mutations listed in Table C3.2. Certain mutations mapped in genes related to the bacterial cell envelope: *rlpB, yejM, dipZ, yhbG.* This finding is in agreement with considerations made above and also with literature data that underline the relevance of the envelope as a barrier [33,38,45,47,123,164]. Curiously enough, one of the mutations, in the gene *rlpB*, involved in LPS transport and induced by bile [131], had been previously described by in a study that selected bile resistant-mutants *in vitro* [88]. LPS seemed to be altered in another mutant isolated from the murine gallbladder, which carried a mutation in the poorly known *yejM* gene, which is also induced by bile [131]. In *E. coli, yejM* may be related to cell permeability and lipid A production [132,133]. Lipid A is one of the three structural regions of LPS, and it is a highly conserved hydrophobic molecule that serves as an anchor to the membrane for the rest of the LPS [70].

Other mutations that may membrane alterations were detected in bile-resistant isolates from the gall bladder. One mapped in the gene encoding in DsdB, a cytoplasmic membrane protein that could play a role in copper tolerance [136]. Another mutant carried a mutation in *yhbG*, a gene induced by bile [131] which is predicted to encode an ABC transporter [143,144]. In *E. coli*, this gene is essential and plays a role in cell envelope integrity [142].

Mutations in the division factor genes *ftsQ* and *ftsK* were also found in the gallblader isolates. Interestingly, transcription of these genes is activated by bile [131]. In *E. coli*, FtsQ and FtsK belong to a set of nine proteins that participate in septum formation [138,139]. Interestingly, previous studies from our laboratory identified another cell division factor (ZapB) that is induced by bile salts [88].

Altogether, the investigations presented in this study provide a mechanistic model to understand adaptation to bile, a phenomenon that may be fundamental during systemic

infection by *Salmonella enterica*, and perhaps during chronic infection as well.

CONCLUSIONS

1. Genetic analysis provides evidence that the AcrAB efflux system is the only cell function essential for adaptation of *Salmonella enterica* serovar Typhimurium to bile.
2. Impairment of AcrAB in bile-resistant isolates carrying mutations that alter the cell envelope renders these strains bile-sensitive, thus emphasizing the contribution to bile resistance of both the envelope and active efflux.
3. Bile and sodium deoxycholate induce the expression of *acrAB,* and this activation is crucial at early stages of adaptation. However, sustained activity, rather than enhanced gene expression, may be sufficient to confer bile resistance as soon as a critical amount of AcrAB is present in the cell.
4. Populations of *S. enterica* isolated from the gallbladder of BALB/c mice are mostly made of cells that do not carry bile-resistant mutations, indicating that non mutational adaptation is more frequent than mutation during colonization of the gallbladder by *Salmonella.*
5. Whole-genome sequencing of bile-resistant mutants isolated from BALB/c mice gallbladders reveals that most mutations that confer resistance to bile may affect the cellular envelope.

REFERENCES

1. Tindall, B. J., Grimont, P. A. D., Garrity, G. M. & Euz??by, J. P. Nomenclature and taxonomy of the genus Salmonella. *Int. J. Syst. Evol. Microbiol.* **55,** 521–524 (2005).
2. Grimont, P. A. D. & Weill, F.-X. Antigenic formulas of the Salmonella serovars. 7th revision. *WHO Collab. Cent. Ref. Res. Salmonella. Inst. Pasteur* **9,** 1–166 (2007).
3. Popoff, M. Y. & Le Minor, L. Antigenic formulas of the Salmonella serovars 8th ed. World Health Organization Collaborating Center for Reference and Research on Salmonella. *Res Microbiol* **152,** 907–909 (2001).
4. McQuiston, J. R. *et al.* Sequencing and Comparative Analysis of Flagellin Genes fliC, fljB, and flpA from Salmonella. *J. Clin. Microbiol.* **42,** 1923–1932 (2004).
5. Popoff, M. Y., Bockemühl, J. & Gheesling, L. L. Supplement 2002 (no. 46) to the Kauffmann-White scheme. *Res. Microbiol.* **155,** 568–570 (2004).
6. McClelland, M. *et al.* Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. *Nature* **413,** 852–6 (2001).
7. Baumler, A. J., Tsolis, R. M., Ficht, T. A. & Adams, L. G. Evolution of host adaptation in Salmonella enterica. *Infect. Immun.* **66,** 4579–4587 (1998).
8. Baumler, A. & Fang, F. C. Host specificity of bacterial pathogens. *Cold Spring Harb. Perspect. Med.* **3,** 1–20 (2013).
9. Velge, P. *et al.* Multiplicity of Salmonella entry mechanisms, a new paradigm for Salmonella pathogenesis. *Microbiologyopen* **1,** 243–258 (2012).
10. Santos, R. L. *et al.* Animal models of Salmonella infections: Enteritis versus typhoid fever. *Microbes Infect.* **3,** 1335–1344 (2001).
11. Ochman, H. & Wilson, A. C. Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. *J. Mol. Evol.* **26,** 74–86 (1987).
12. Porwollik, S. & McClelland, M. Lateral gene transfer in Salmonella. *Microbes Infect.* **5,** 977–989 (2003).
13. Groisman, E. A. & Ochman, H. How Salmonella became a pathogen. *Trends Microbiol.* **5,** 343–349 (1997).
14. Kelly, B. G., Vespermann, A. & Bolton, D. J. The role of horizontal gene transfer in the evolution of selected foodborne bacterial pathogens. *Food Chem. Toxicol.* **47,** 951–968 (2009).
15. Hensel, M. Evolution of pathogenicity islands of Salmonella enterica. *Int. J. Med. Microbiol.* **294,** 95–102 (2004).
16. Ellermeier, C. D. & Slauch, J. in *The Prokaryotes* (eds. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. & Stackebrandt, E.) 123–158 (Springer, 2006). at <file:///Users/melinaki/Documents/Papersoldmac/Papers/2014/Stieglmeier/Unknown/2014 Stieglmeier.pdf\npapers://dfa503e6-d258-4223-9ef8-d69bedc66f2d/Paper/p8237>
17. Que, F., Wu, S. & Huang, R. Salmonella pathogenicity island 1(SPI-1) at work. *Curr. Microbiol.* **66,** 582–587 (2013).
18. Ochman, H., Soncini, F. C., Solomon, F. & Groisman, E. A. Identification of a pathogenicity island required for Salmonella survival in host cells. *Proc. Natl. Acad. Sci.* **93,** 7800–7804 (1996).
19. Humphries, A. D. *et al.* Role of fimbriae as antigens and intestinal colonization factors of Salmonella serovars. *FEMS Microbiol. Lett.* **201,** 121–125 (2001).
20. Haraga, A., Ohlson, M. B. & Miller, S. I. Salmonellae interplay with host cells.

Nat. Rev. Microbiol. **6,** 53–66 (2008).
21. Zhou, D. & Galán, J. Salmonella entry into host cells : the work in concert of type III secreted effector proteins. *Microbes Infect.* 1293–1298 (2001).
22. Zhang, S. *et al.* MINIREVIEW Molecular Pathogenesis of Salmonella enterica Serotype typhimurium-induced diarrhea. *Infect. Immun.* **71,** 1–12 (2003).
23. Bäumler, A. J., Winter, S. E., Thiennimitr, P. & Casadesús, J. Minireview Intestinal and chronic infections : Salmonella lifestyles. *Environ. Microbiol. Rep.* **3,** 508–517 (2011).
24. Watson, K. G. & Holden, D. W. Microreview Dynamics of growth and dissemination of Salmonella in vivo. *Cell. Microbiol.* **12,** 1389–1397 (2010).
25. Tischler, A. D. & Mckinney, J. D. Contrasting persistence strategies in Salmonella and Mycobacterium. *Curr. Opin. Microbiol.* **4,** 93–99 (2010).
26. Monack, D. M., Bouley, D. M. & Falkow, S. Salmonella typhimurium Persists within Macrophages in the Mesenteric Lymph Nodes of Chronically Infected Nramp1 ε / ε Mice and Can Be Reactivated by IFN ″ Neutralization. *J. Exp. Med.* **199,** (2004).
27. Lai, C. W., Chan, R. C., Cheng, A. F., Sung, J. Y. & Leung, J. W. Common bile duct stones: a cause of chronic salmonellosis. *Am. J. Gastroenterol.* **87,** 1198–1199 (1992).
28. Prouty, A. M., Schwesinger, W. H. & Gunn, J. S. Biofilm Formation and Interaction with the Surfaces of Gallstones by Salmonella spp . *Infect. Immun.* **70,** 2640–2649 (2002).
29. Crawford, R. W. *et al.* Gallstones play a signi fi cant role in Salmonella spp . gallbladder colonization and carriage. *Proc. Natl. Acad. Sci.* **107,** 4353–4358 (2010).
30. Rychlik, I. & Barrow, P. A. Salmonella stress management and its relevance to behaviour during intestinal colonisation and infection. *FEMS Microbiol. Rev.* **29,** 1021–1040 (2005).
31. Clements, M., Eriksson, S., Tezcan-merdol, D. & Hinton, J. C. D. Virulence gene regulation in Salmonella enterica. 178–185 (2001).
32. Finlay, B. B. & Falkow, S. MicroReview Salmonella as an intracellular parasite. *Mol. Microbiol.* **3,** 1833–1841 (1989).
33. Alvarez-Ordonez, A. *et al.* Salmonella spp . survival strategies within the host gastrointestinal tract. *Food Res. Int.* 3268–3281 (2016). doi:10.1099/mic.0.050351-0
34. Spector, M. P. & Kenyon, W. J. Resistance and survival strategies of Salmonella enterica to environmental stresses. *FRIN* (2011). doi:10.1016/j.foodres.2011.06.056
35. Fontana, J. *et al.* Functions of cells and human body. Multimedia textbook. at <http://fblt.cz/en/>
36. Hofmann, A. F. Bile Acids : The Good , the Bad , and the Ugly. *News Physiol. Sci.* (1999).
37. Hofmann, A. F. Bile Secretion in Mice and Men. *Hepatology* 847–852 (2001). doi:10.1053/jhep.2001.28191
38. Begley, M., Gahan, C. G. M. & Hill, C. The interaction between bacteria and bile. *FEMS Microbiol. Rev.* **29,** 625–651 (2005).
39. Casadesús, J., Hernández, S. B., Cota, I. & Ramos-Morales, F. in *The Lure of*

Bacterial Genetics (eds. Casadesús, J., Hughes, K. T. & Maloy, S.) 153–162 (American Society of Microbiology, 2011). doi:10.1128/9781555816810

40. Hofmann, A. F. & Hagey, L. R. Bile acids: Chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell. Mol. Life Sci.* **65,** 2461–2483 (2008).
41. Russell, D. W. The enzymes, regulation, and genetics of bile acid synthesis. *Annu. Rev. Biochem.* **72,** 137–174 (2003).
42. Monte, M. J., Marin, J. J. G., Antelo, A. & Vazquez-Tato, J. Bile acids: Chemistry, physiology, and pathophysiology. *World J. Gastroenterol.* **15,** 804–816 (2009).
43. Ridlon, J. M., Kang, D.-J. & Hylemon, P. B. Bile salt biotransformations by human intestinal bacteria. *J. Lipid Res.* **47,** 241–59 (2006).
44. Hylemon, P. B. & Harder, J. Biotransformation of monoterpenes, bile acids, and other isoprenoids in anaerobic ecosystems. *FEMS Microbiol. Rev.* **22,** 475–488 (1998).
45. Merritt, M. E. & Donaldson, J. R. Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *J. Med. Microbiol.* **58,** 1533–1541 (2009).
46. Hofmann, A. F. & Eckmann, L. How bile acids confer gut mucosal protection against bacteria. *Pnas* **103,** 4333–4334 (2006).
47. Gunn, J. S. Mechanisms of bacterial resistance and response to bile. 907–913 (2000).
48. Leverrier, P. *et al.* Susceptibility and Adaptive Response to Bile Salts in Propionibacterium freudenreichii : Physiological and Proteomic Analysis Susceptibility and Adaptive Response to Bile Salts in Propionibacterium freudenreichii : Physiological and Proteomic Analysis. *Appl. Environ. Microbiol.* **69,** 3809–3818 (2003).
49. Fujisawa, T. & Mori, M. Influence of bile salts on β-glucuronidase activity of intestinal bacteria. *Lett. Appl. Microbiol.* **22,** 271–274 (1996).
50. Noh, D. O. & Gilliland, S. E. Influence of bile on cellular integrity and beta-galactosidase activity of Lactobacillus acidophilus. *J. Dairy Sci.* **76,** 1253–1259 (1993).
51. Coleman, R., Lowe, P. J. & Billington, D. Membrane lipid composition and susceptibility to bile salt damage. *BBA - Biomembr.* **599,** 294–300 (1980).
52. Gómez Zavaglia, A., Kociubinski, G., Pérez, P., Disalvo, E. & De Antoni, G. Effect of bile on the lipid composition and surface properties of bifidobacteria. *J. Appl. Microbiol.* **93,** 794–799 (2002).
53. Záratea, G., Gonzaleza, S., Chaia, A. P. & Oliver, G. Effect of bile on the β - galactosidase activity of dairy propionibacteria. *Lait* **80,** 267–276 (2000).
54. Hofmann, M. *et al.* LUV's lipid composition modulates diffusion of bile acids. *Chem. Phys. Lipids* **110,** 165–171 (2001).
55. Cabral, D. J., Small, D. M., Lilly, H. S. & Hamilton, J. A. Transbilayer movement of bile acids in model membranes. *Biochemistry* **26,** 1801–1804 (1987).
56. Schubert, R., Jaroni, H., Schoelmerich, J. & Schmidt, K. H. Studies on the Mechanism of Bile Salt-Induced Liposomal Membrane Damage. *Digestion* **28,** 181–190 (1983).
57. Chou, C. & Cheng, S. Recovery of low-temperature stressed E . coli O157 : H7 and its susceptibility to crystal violet , bile salt , sodium chloride and ethanol. **61,** 127–136 (2000).
58. Chou, L. S. & Weimer, B. Isolation and characterization of acid- and bile-tolerant

isolates from strains of Lactobacillus acidophilus. *J. Dairy Sci.* **82,** 23–31 (1999).
59. King, T., Ferenci, T. & Szabo, E. A. The effect of growth atmosphere on the ability of Listeria monocytogenes to survive exposure to acid, proteolytic enzymes and bile salts. *Int. J. Food Microbiol.* **84,** 133—143 (2003).
60. Fernández Murga, M. L., Bernik, D., Font de Valdez, G. & Disalvo, a E. Permeability and stability properties of membranes formed by lipids extracted from Lactobacillus acidophilus grown at different temperatures. *Arch. Biochem. Biophys.* **364,** 115–21 (1999).
61. Bernstein, C., Bernstein, H., Payne, C. M., Beard, S. E. & Schneider, J. Bile salt activation of stress response promoters in Escherichia coli. *Curr. Microbiol.* **39,** 68–72 (1999).
62. Chou, J. H., Greenberg, J. T. & Demple, B. Posttranscriptional repression of Escherichia coli OmpF protein in response to redox stress: Positive control of the micF antisense RNA by the soxRS locus. *J. Bacteriol.* **175,** 1026–1031 (1993).
63. Prieto, A. I., Ramos-Morales, F. & Casadesús, J. Bile-induced DNA damage in Salmonella enterica. *Genetics* **168,** 1787–1794 (2004).
64. Prieto, A. I., Ramos-Morales, F. & Casadesús, J. Repair of DNA damage induced by bile salts in Salmonella enterica. *Genetics* **174,** 575–584 (2006).
65. Gahan, C. G. M., O'Mahony, J. & Hill, C. Characterization of the groESL operon in Listeria monocytogenes: Utilization of two reporter systems (gfp and hly) for evaluating in vivo expression. *Infect. Immun.* **69,** 3924–3932 (2001).
66. Rince, A., Flahaut, S. & Auffray, Y. Identification of general stress genes in Enterococcus faecalis. *Int. J. Food Microbiol.* **55,** 87–91 (2000).
67. Schmidt, G. & Zink, R. Basic features of the stress response in three species of bifidobacteria: B. longum, B. adolescentis, and B. breve. *Int. J. Food Microbiol.* **55,** 41–45 (2000).
68. T. J. Silhavy, D. Kahne, and S. W. The bacterial cell envelope. *Cold Spring Harb. Perspect. Biol.* **2,** 1–17 (2010).
69. Lerouge, I. & Vanderleyden, J. O-antigen structural variation : mechanisms and possible roles in animal / plant ^ microbe interactions. *FEMS Microbiol. Rev.* **26,** (2001).
70. Raetz, C. R. H. & Whitfield, C. L <scp>IPOPOLYSACCHARIDE</scp> E <scp>NDOTOXINS</scp>. *Annu. Rev. Biochem.* **71,** 635–700 (2002).
71. Picken, R. & Beacham, I. Bacteriophage-resistant mutants of Escherichia coli K12. Location of Receptors within the Lipopolysaccharide. *J. Gen. Microbiol.* 305–318 (1977).
72. Crawford, R. W. *et al.* Very Long O-antigen Chains Enhance Fitness during Salmonella-induced Colitis by Increasing Bile Resistance. *PLoS Pathog.* **8,** (2012).
73. Ramos-morales, F., Prieto, A. I., Beuzon, C. R., Holden, D. W. & Casadesús, J. Role for Salmonella enterica Enterobacterial Common Antigen in Bile Resistance and Virulence. *J. Bacteriol.* **185,** 5328–5332 (2003).
74. Thanassi, D. G., Cheng, L. W. & Nikaido, H. Active efflux of bile salts by Escherichia coli . Active Efflux of Bile Salts by Escherichia coli. **179,** 2512–2518 (1997).
75. Horiyama, T., Yamaguchi, A. & Nishino, K. TolC dependency of multidrug efflux systems in Salmonella enterica serovar Typhimurium. *J. Antimicrob. Chemother.* **65,** 1372–1376 (2010).

76. Ma, D. *et al.* Genes acrA and acrB encode a stress-induced efflux system of Escherichia coli. *Mol. Microbiol.* **16,** 45–55 (1995).
77. Ma, D. *et al.* Molecular Cloning and Characterization of acrA and acrE Genes of Escherichia coli. **175,** 6299–6313 (1993).
78. Zgurskaya, H. I. & Nikaido, H. Bypassing the periplasm : Reconstitution of the AcrAB multidrug efflux pump of Escherichia coli. *Proc. Natl. Acad. Sci.* **96,** 7190–7195 (1999).
79. Nikaido, E., Yamaguchi, A. & Nishino, K. AcrAB Multidrug Efflux Pump Regulation in Salmonella enterica serovar Typhimurium by RamA in Response to Environmental Signals *. **283,** 24245–24253 (2008).
80. Murakami, S., Nakashima, R., Yamashita, E. & Yamaguchi, A. Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature* **419,** 587–593 (2002).
81. Nishino, K., Latifi, T. & Groisman, E. A. Virulence and drug resistance roles of multidrug efflux systems of Salmonella enterica serovar Typhimurium. *Mol. Microbiol.* **59,** 126–141 (2006).
82. Pos, K. M. Trinity revealed: Stoichiometric complex assembly of a bacterial multidrug efflux pump. *Proc. Natl. Acad. Sci.* **106 ,** 6893–6894 (2009).
83. Pos, K. M. Biochimica et Biophysica Acta Drug transport mechanism of the AcrB ef fl ux pump. *BBA - Proteins Proteomics* **1794,** 782–793 (2009).
84. Baucheron, S. *et al.* Bile-mediated activation of the acrAB and tolC multidrug efflux genes occurs mainly through transcriptional derepression of ramA in Salmonella enterica serovar Typhimurium. **1,** 2400–2406 (2014).
85. Cano, D. A., Pucciarelli, M. G., Garc??a-Del Portillo, F. & Casades??s, J. Role of the RecBCD recombination pathway in Salmonella virulence. *J. Bacteriol.* **184,** 592–595 (2002).
86. van Velkinburgh, J. C. & Gunn, J. S. PhoP-PhoQ-Regulated Loci Are Required for Enhanced Bile Resistance in Salmonella spp . **67,** 1614–1622 (1999).
87. Prouty, A. M. *et al.* Transcriptional regulation of Salmonella enterica serovar Typhimurium genes by bile. **41,** 177–185 (2004).
88. Hernandez, S. B., Cota, I., Ducret, A., Aussel, L. & Casadesus, J. Adaptation and Preadaptation of Salmonella enterica to Bile. **8,** (2012).
89. Schmieger, H. Phage P22-mutants with increased or decreased transduction abilities. *MGG Mol. Gen. Genet.* **119,** 75–88 (1972).
90. Smith, H. O. & Levine, M. Two sequential repressions of DNA synthesis in the stablishment of lysogeny by phage P22 and its mutants. *Proc. Natl. Acad. Sci.* **52,** 356–363 (1964).
91. Cherepanov, P. P. & Wackernagel, W. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158,** 9–14 (1995).
92. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. **2000,** (2000).
93. Bolivar, F. & Backman, K. Plasmids of Escherichia coli as cloning vectors. *Methods Enzymol.* **68,** 245–267 (1979).
94. Inoue, H., Nojima, H. & Okayama, H. High efficiency transformation of Escherichia coli with plasmids. *Gene* **96,** 23–28 (1990).
95. Rappleye, C. A. & Roth, J. R. A Tn 10 Derivative (T-POP) for Isolation of Insertions with Conditional (Tetracycline-Dependent) Phenotypes. **179,** 5827–

5834 (1997).
96. Lee, C., Wozniak, C., Karlinsey, J. E. & Hughes, K. T. Genomic Screening for Regulatory Genes Using the T-POP Transposon. *Methods in Enzymology* **421,** 159–167 (2007).
97. Kleckner, N., Bender, J. & Gottesman, S. Uses of transposons with emphasis on Tn10. *Methods Enzymol.* **204,** 139–180 (1991).
98. Chun, K. T., Edenberg, H. J., Kelley, M. R. & Goebl, M. G. Rapid Amplification of Uncharacterized Transposon- tagged DNA Sequences from Genomic DNA. *DNA Seq.* **240,** 233–240 (1997).
99. Szybalski, W. & Bryson, V. Genetic Studies on Microbial Cross Resistance To Toxic Agents. *J. Bacteriol.* **64,** 489–499 (1952).
100. Rothberg, J. M. & Leamon, J. H. The development and impact of 454 sequencing. *Nat. Biotechnol.* **26,** 1117–1124 (2008).
101. Roche Diagnostic. Rapid Library Preparation Method Manual GS FLX+ Series - XL+. *Roche Diagnostic* (2011).
102. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25,** 1754–1760 (2009).
103. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26,** 589–595 (2010).
104. Viveiros, M. *et al.* Demonstration of intrinsic efflux activity of Escherichia coli K-12 AG100 by an automated ethidium bromide method. *Int. J. Antimicrob. Agents* **31,** 458–462 (2008).
105. Martins, M. *et al.* An instrument-free method for the demonstration of efflux pump activity of bacteria. *In Vivo (Brooklyn).* **20,** 657–664 (2006).
106. Marinus, M. G. & Løbner-Olesen, A. DNA Methylation. *EcoSal Plus* **6,** 1–62 (2014).
107. Løbner-olesen, A., Skovgaard, O. & Marinus, M. G. Dam methylation : coordinating cellular processes. *Curr. Opin. Microbiol.* 154–160 (2005). doi:10.1016/j.mib.2005.02.009
108. Low, D. A., Weyand, N. J. & Mahan, M. J. Roles of DNA Adenine Methylation in Regulating Bacterial Gene Expression and Virulence. *Infect. Immun.* **69,** 7197–7204 (2001).
109. Torreblanca, J. & Casadesús, J. DNA Adenine Methylase Mutants of Salmonella Eylbhimurium and a Novel Dam-Regulated Locus. *Genetics* **26,** 15–26 (1996).
110. Arends, S. J. R. *et al.* Discovery and Characterization of Three New Escherichia coli Septal Ring Proteins That Contain a SPOR Domain : DamX , DedD , and RlpA □. *J. Bacteriol.* **192,** 242–255 (2010).
111. Lopez-Garrido, J., Cheng, N., Garcia-Quintanilla, F., García-del Portillo, F. & Casadesús, J. Identification of the Salmonella enterica damX Gene Product , an Inner Membrane Protein Involved in Bile Resistance □ †. *J. Bacteriol.* **192,** 893–895 (2010).
112. Hancock, R. E. W. & McPhee, J. B. Salmonella ' s Sensor for Host Defense Molecules. *Cell* 320–323 (2005). doi:10.1016/j.cell.2005.07.021
113. Gunn, J. S. Bacterial modification of LPS and resistance to antimicrobial peptides. *J. Endotoxin Res.* **7,** 57–62 (2001).
114. Nickerson, C. A. & Curtiss III, R. Role of Sigma Factor RpoS in Initial Stages of Salmonella typhimurium Infection. *Infect. Immun.* **65,** 1814–1823 (1997).

115. Chen, C. *et al.* Central Regulatory Role for the RpoS Sigma Factor in Expression of Salmonella dublin Plasmid Virulence Genes. *J. Bacteriol.* **177,** 5303–5309 (1995).
116. Glauner, B., Holtje, J.-V. & Schwarz, U. The Composition of the Murein of Escherichia coli *. *J. Biol. Chem.* **263,** 10088–10095 (1988).
117. Koronakis, V. TolC - the bacterial exit duct for proteins and drugs. *FEBS Lett.* **555,** 66–71 (2003).
118. Nishino, K., Nikaido, E. & Yamaguchi, A. Regulation and physiological function of multidrug ef fl ux pumps in Escherichia coli and Salmonella. *BBA - Proteins Proteomics* **1794,** 834–843 (2009).
119. Broadbent, S. E., Balbontin, R., Casadesus, J., Marinus, M. G. & Woude, M. Van Der. YhdJ , a Nonessential CcrM-Like DNA Methyltransferase of Escherichia coli and Salmonella enterica □. *J. Bacteriol.* **189,** 4325–4327 (2007).
120. Ramos, J. L. *et al.* The TetR Family of Transcriptional Repressors. **69,** 326–356 (2005).
121. Frisch, R. L. *et al.* Separate DNA Pol II- and Pol IV-Dependent Pathways of Stress-Induced Mutation during Double-Strand-Break Repair in Escherichia coli Are Controlled by RpoS □. *J. Bacteriol.* **192,** 4694–4700 (2010).
122. Prouty, A. M., Brodsky, I. E., Falkow, S. & Gunn, J. S. Bile-salt-mediated induction of antimicrobial and bile resistance in Salmonella typhimurium. *Microbiology* 775–783 (2004). doi:10.1099/mic.0.26769-0
123. Prieto, A. I. *et al.* Roles of the Outer Membrane Protein AsmA of Salmonella enterica in the Control of marRAB Expression and Invasion of Epithelial Cells □. **191,** 3615–3622 (2009).
124. Hernández, S. B., Ayala, J. A., Rico-pérez, G., García-del Portillo, F. & Casadesús, J. Increased bile resistance in Salmonella enterica mutants lacking Prc periplasmic protease. 87–92 (2013). doi:10.2436/20.1501.01.183
125. Usui, M., Asai, T. & Sato, S. Low expression of AcrB in the deoxycholate-sensitive strains of Salmonella enterica subspecies enterica serovar Pullorum. 366–368 (2011). doi:10.1111/j.1348-0421.2011.00321.x
126. Viveiros, M. *et al.* Demonstration of intrinsic efflux activity of Escherichia coli K-12 AG100 by an automated ethidium bromide method (PDF Download Available).pdf. *Int. J. Antimicrob. Agents* 458–462 (2008).
127. Sánchez-romero, M. A. & Casadesús, J. Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. **111,** 355–360 (2014).
128. Canonne-Hergaux, F., Gruenheid, S., Govoni, G. & Gros, P. The Nramp1 protein and its role in resistance to infection and macrophage function. *Proc. Assoc. Am. Physicians* **111,** 283–289 (1999).
129. Gruenheid, S., Pinner, E., Desjardins, M. & Gros, P. Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. *J. Exp. Med.* **185,** 717–730 (1997).
130. Wu, T. *et al.* Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 11754–11759 (2006).
131. Kr??ger, C. *et al.* An infection-relevant transcriptomic compendium for salmonella enterica serovar typhimurium. *Cell Host Microbe* **14,** 683–695 (2013).
132. Hirvas, L., Nurminen, M., Helander, I. M., Vuorio, R. & Vaara, M. The lipid a

biosynthesis deficiency of the Escherichia coli antibiotic-supersensitive mutant LH530 is suppressed by a novel locus, ORF195. *Microbiology* **143,** 73–81 (1997).
133. De Lay, N. R. & Cronan, J. E. Genetic interaction between the Escherichia coli AcpT phosphopantetheinyl transferase and the YejM inner membrane protein. *Genetics* **178,** 1327–1337 (2008).
134. Stewart, E. J., Katzen, F. & Beckwith, J. Six conserved cysteines of the membrane protein DsbD are required for the transfer of electrons from the cytoplasm to the periplasm of Escherichia coli. *EMBO J.* **18,** 5963–5971 (1999).
135. Gordon, E. H., Page, M. D., Willis, A. C. & Ferguson, S. J. Escherichia coli DipZ: anatomy of a transmembrane protein disulphide reductase in which three pairs of cysteine residues, one in each of three domains, contribute differentially to function. *Mol. Microbiol.* **35,** 1360–1374 (2000).
136. Gupta, S. D., Wu, H. C., Rick, P. D. & Rick, P. D. A Salmonella typhimurium genetic locus which confers copper tolerance on copper-sensitive mutants of Escherichia coli A Salmonella typhimurium Genetic Locus Which Confers Copper Tolerance on Copper-Sensitive Mutants of Escherichia coli †. **179,** 4977–4984 (1997).
137. Blattner, F. R. *et al.* The Complete Genome Sequence of Escherichia coli K-12. *Science (80-.).* **277,** 1453–1462 (1997).
138. Chen, J. C. & Beckwith, J. FtsQ, FtsL and Ftsl require FtsK, but not FtsN, for co-localization with FtsZ during Escherichia coli cell division. *Mol. Microbiol.* **42,** 395–413 (2001).
139. Buddelmeijer, N. & Beckwith, J. A complex of the Escherichia coli cell division proteins FtsL, FtsB and FtsQ forms independently of its localization to the septal region. *Mol. Microbiol.* **52,** 1315–1327 (2004).
140. Heithoff, D. M. *et al.* Salmonella DNA adenine methylase mutants confer cross-protective immunity. *Infect. Immun.* **69,** 6725–6730 (2001).
141. Giacomodonato, M. N., Sarnacki, S. H., Caccuri, R. L., Sordelli, D. O. & Cerquetti, M. C. Host response to a dam mutant of Salmonella enterica serovar enteritidis with a temperature-sensitive phenotype. *Infect. Immun.* **72,** 5498–5501 (2004).
142. Sperandeo, P., Pozzi, C., Dehò, G. & Polissi, A. Non-essential KDO biosynthesis and new essential cell envelope biogenesis genes in the Escherichia coli yrbG – yhbG locus. **157,** 547–558 (2006).
143. Dassa, E. & Bouige, P. The ABC of ABCS: a phylogenetic and functional classification of ABC systems in living organisms. *Res. Microbiol.* **152,** 211–229 (2001).
144. Saurin, W., Hofnung, M. & Dassa, E. Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. *J. Mol. Evol.* **48,** 22–41 (1999).
145. Blair, J. M. & Piddock, L. J. Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. *Curr. Opin. Microbiol.* **12,** 512–519 (2009).
146. Paulsen, I. T., Park, J. H., Choi, P. S. & Jr., M. H. S. A family of Gram-negative bacterial outer membrne factors that function in the export of protiens, carbohydrates, drugs and heavy metals from Gram-negative bacteria. *FEMS Microbiol. Lett* **156,** 1–8 (1997).
147. Nikaido, H. Multidrug efflux pumps of gram-negative bacteria. *J. Bacteriol.* **178,**

5853–5859 (1996).
148. Nikaido, H. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Semin. Cell Dev. Biol.* **12,** 215–223 (2001).
149. Lacroix, F. J. C. *et al.* Salmonella typhimurium acrB-like gene: Identification and role in resistance to biliary salts and detergents and in murine infection. *FEMS Microbiol. Lett.* **135,** 161–167 (1996).
150. Nikaido, H., Basina, M., Nguyen, V., Emiko, Y. & Rosenberg, E. Y. Multidrug Efflux Pump AcrAB of Salmonella typhimurium Excretes Only Those β -Lactam Antibiotics Containing Lipophilic Side Chains Multidrug Efflux Pump AcrAB of Salmonella typhimurium Excretes Only Those NL -Lactam Antibiotics Containing Lipophilic Side Ch. **180,** 4686–4692 (1998).
151. Prouty, A. M., Van Velkinburgh, J. C. & Gunn, J. S. Salmonella enterica serovar typhimurium resistance to bile: Identification and characterization of the tolQRA cluster. *J. Bacteriol.* **184,** 1270–1276 (2002).
152. Nishino, K., Hayashi-Nishino, M. & Yamaguchi, A. H-NS modulates multidrug resistance of Salmonella enterica serovar typhimurium by repressing multidrug efflux genes acrEF. *Antimicrob. Agents Chemother.* **53,** 3541–3543 (2009).
153. Olliver, A., Vallé, M., Chaslus-Dancla, E. & Cloeckaert, A. Overexpression of the multidrug efflux operon acrEF by insertional activation with IS1 or IS10 elements in Salmonella enterica serovar typhimurium DT204 acrB mutants selected with fluoroquinolones. *Antimicrob. Agents Chemother.* **49,** 289–301 (2005).
154. Li, X., Plésiat, P. & Nikaido, H. The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria. *Clin. Microbiol. Rev.* **28,** 337–418 (2015).
155. Tsukagoshi, N. & Aono, R. Entry into and release of solvents by Escherichia coli in an organic-aqueous two-liquid-phase system and substrate specificity of the AcrAB-TolC solvent-extruding pump. *J. Bacteriol.* **182,** 4803–4810 (2000).
156. Lennen, R. M., Politz, M. G., Kruziki, M. A. & Pfleger, B. F. Identification of transport proteins involved in free fatty acid efflux in Escherichia coli. *J. Bacteriol.* **195,** 135–144 (2013).
157. Nikaido, H. MINI-REVIEWS The role of outer membrane and efflux pumps in the resistance of Can we improve drug. 93–98 (1998).
158. Nikaido, H. Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrob. Agents Chemother.* **33,** 1831–1836 (1989).
159. Wandersman, C. Secretion across the bacterial outer membrane. *Trends Genet.* **8,** 317–322 (1992).
160. Zgurskaya, H. I. & Nikaido, H. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol Microbiol* **37,** 219–225 (2000).
161. Garai, P., Gnanadhas, D. P. & Chakravortty, D. Salmonella enterica serovars Typhimurium and Typhi as model organisms: Revealing paradigm of host-pathogen interactions. *Virulence* **3,** 377–388 (2012).
162. Gunn, J. S. *et al.* Salmonella chronic carriage: Epidemiology, diagnosis, and gallbladder persistence. *Trends Microbiol.* **22,** 648–655 (2014).
163. Gonzalez-Escobedo, G., Marshall, J. M. & Gunn, J. S. Chronic and acute infection of the gall bladder by Salmonella Typhi: understanding the carrier state. *Nat Rev Micro* **9,** 9–14 (2011).
164. Raivio, T. L. Envelope stress responses and Gram-negative bacterial pathogenesis. *Mol. Microbiol.* **56,** 1119–1128 (2005).